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Chitin and chitosan as reliable templates towards the carbohydrate backbone of bacterial peptidoglycan

Dissertação para obtenção do Grau de Doutor em
Química Sustentável

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À Nídia, à Pilar

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Abstract

Peptidoglycan (PGN) is a major component of the bacteria cell wall that surrounds and protect bacteria from the surrounding environment. The composition of PGN is often linked with the outcome of bacterial infections and its synthesis is the target of the different classes of antibiotics frequently used in the treatment of these bacterial infections.

The scientific community has been requesting to availability of increasing amounts of pure, well defined, PGN fragments in order to be able to move forward with the biological studies in a reliable way. The extraction processes used to purify PGN from bacteria rely on the use of harsh conditions that can be harmful to the user and also the environment.

Thus, through the last thirty years, different groups have been dedicating time and resources to establish new synthetic routes to produce PGN fragments of different composition. These routes rely on the use of fully functionalized glucosamine building blocks, through a protecting group orthogonal synthesis, followed by glycosylation and peptide coupling reactions.

The challenge, fully framed in a sustainable chemistry doctoral program, was to develop new synthetic routes towards PGN fragments using more sustainable processes. In this thesis it is reported the synthesis of PGN fragments using as starting material the polymers of chitin and chitosan, which are present in significant amount in food industry wastes.

In the first approach, an acetolysis reaction of chitin was used to give a peracetylated disaccharide, in a gram scale amount, which, through protecting group orthogonal synthesis, can give an advanced PGN intermediate in a five synthetic steps. As this intermediate possesses all the functional groups in the correct position as the PGN fragments, it may be considered a high value synthetic intermediate.

On the second approach, the polymer of chitosan was used as starting material in a chemoenzymatic synthesis. Through orthogonal protecting group strategy, it was possible modify a high weight chitosan molecules in order to produce a PGN surrogate. Using commercially available PGN hydrolases, we were able to hydrolyze this surrogate in order to identify the oligosaccharides that are present in the PGN sugar backbone.

Key-words: Peptidoglycan, Sustainable Chemistry, Chitin, Chitosan

Resumo

O Peptidoglicano (PGN) é um importante constituinte da parede celular bacteriana que está diretamente relacionado com a progressão de diferentes infecções bacterianas e com diferentes mecanismos de resistência a antibióticos.

Este trabalho surge no enquadramento do problema com o qual a comunidade científica se tem vindo a deparar aquando da obtenção de fragmentos de PGN puros e, em quantidade, que permita a realização de ensaios biológicos. Tendo em conta que, os processos de extração da parede celular da bactéria, utilizam condições agressivas para o utilizador bem como, para com o meio ambiente.

Desta forma, a comunidade científica tem vindo a desenvolver, ao longo dos últimos trinta anos, novas vias de síntese destes fragmentos. Estas vias sintéticas baseiam-se na utilização de unidades de glucosamina funcionalizadas que através do uso de grupos protectores, síntese ortogonal, reacções de glicosilação e acoplamento peptídico, e dão origem a fragmentos de PGN.

Sendo este projecto inserido num programa doutoral em química sustentável o desafio era criar novas vias sintéticas para obtenção de fragmentos de PGN utilizando, em alternativa aos métodos já reportados, processos mais sustentáveis.

Assim sendo nesta tese é apresentada a síntese de fragmentos de PGN utilizando como material de partida, um desperdício da indústria alimentar, quitina e quitosano.

Numa primeira abordagem através de uma reacção de acetólise da quitina foi possível obter o seu correspondente dissacárido per-acetilado que através de uma estratégia de grupos protectores, originou um intermediário avançado em cinco passos de síntese. Este intermediário apresenta todos os grupos funcionais, posicionados corretamente, que os fragmentos de possuem PGN, sendo por isso considerado um intermediário de alto valor sintético.

Numa segunda estratégia, foi utilizado quitosano como material de partida numa síntese quimioenzimatática. Através de uma estratégia de grupos portectores foi possível modificar um polímero de alto peso molecular num mimético a componente de carbohidrato do PGN. Após hidrólise de enzimas que reconhecem o PGN, foi possível identificar vários oligossacáridos que constituem o PGN.

Palavras-chave: Peptidoglicano, Química Sustentável, Quitina, Quitosano

Contents

Index of Figures

Index of Tables

List of Abbreviations

Ac	acetyl
ACN	acetonitrile
Ala	alanine
Alloc	allyloxycarbony
aq.	aqueous
Ar	aryl
Arg	arginine
Bn	benzyl
Boc	*tert*-butyloxycarbonyl
Bu	butyl
C55-P	undecaprenyl pyrophosphate
C55-PP	undecaprenyl pyrodiphosphate
cat.	catalyst
CBz	carboxybenzy
CDI	1,1'-carbonyldiimidazole
COS	chitooligosaccharide
CP	cross polarization
2-CPA	(*S*)-(−)-2-chloropropionic acid
d	doublet
DA	degree of acetylation
DAP	2,6-diaminopimelic acid
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DCE	1,2-dichloroethane
DCM	dichloromethane
DD-TPases	DD-transpeptidases
D*div*	1-(4,4-dimethyl-2,6-dioxocyclohexylidene)isovaleryl
DES	deep eutectic solvent
DMAc	*N*,*N*-dimethylacetamide
DMAP	*N*,*N*-dimethyl-pyridin-4-amine
DMF	*N,N*-dimethylformamide
DMM	2,3-dimethyl maleimide
DMSO	dimethyl sulfoxide
DMTST	dimethyl(methylthio)sulfonium trifluoromethanesulfonate
DP	degree of polymerization
DS	degree of substitution
DTBMP	2,6-di-*tert*-butyl-4-methylpyridine
e.g.	*exempli gratia* (for the sake of example)
EGF	epidermal growth factor
eq	equation
equiv.	equivalent(s)
Et	ethyl

Fmoc	fluorenylmethyloxycarbonyl
FPP	farnesyl pyrophosphate
Fru-6-P	fructose-6-phophate
FtsW-RodA	lipid II flippase
GFAT	glutamine-fructose-6-phostate amindotransferase
GlcN-1-P	glucosamine-1-phosphate
GlcN-6-P	glucosamine-6-phosphate
GlcNAc-1-P	*N*-acetylglucosamine-1-phosphate
GlcNAcases	β–*N*–acetylglucosaminidase
GlmM	phosphoglucosamine mutase
GlmS	glucosamine-6-phosphate synthase
GlmU	*N*-acetylglucosamine-1-phosphate transferase
Glu	glutamic acid
GPC	gel permeation chromatography
GTases	glycosyltransferases
h	hour
HMDS	hexamethyldisilane
HPLC	high performance/pressure liquid chromatography
HSQC	heteronuclear single quantum coherence spectroscopy
Im	imidazole
IR	infrared
J	coupling constant
Lac	D-Lactyl
LC	liquid chromatography
LDA	lithium diisopropylamide
Lig.	ligand
lit.	literature
Lys	lysine
m	multiplet
m	meta
M	molar (concentration)
M.p.	melting point
MALDI	matrix-assisted laser desorption/ionisation
MAS	magic angle spinning
mCPBA	3-chlorobenzoic acid
MDP	N-acetylmuramyl dipeptide
Me	methyl
min	minutes
Mpp	*N*-acetylmuramyl pentapeptide
MraY	phosphor-MurNAc-pentapetide translocase
Ms	mesyl
MS	molecular sieves
MS	mass spectra

Mur	muramyl
MW	microwave
Mw	molecular weight
NAG	*N*-acetyl glucosamine
NAM	*N*-acetyl muramic acid
n.d.	nothing detected
NIS	*N*-Iodosuccionimide
NMR	nuclear magnetic resonance
Nu	nucleophile
o	*ortho*
p	*para*
PA	pattern of acetylation
PBPs	penicillin-binding proteins
PEG	poly(ethylene glycol)
Pg	protecting group
PGN	peptidoglycan
PGRP	peptidoglycan recognition proteins
Ph	phenyl
Phth	phthalimide
PMB	*p*-methoxybenzyl
Pyr	pyridine
quant.	quantitative
r.t.	room temperature
s	singlet
Ser	serine
SMbp	methyl-5-*tert*-butylphenylthiol
t	triplet
t	terciary
TA	teichoic acids
TBAF	tetrabutylammonium fluoride
TBDMS	*tert*-butyldimethylsilane
TBDPS	*tert*-butyldiphenylsilane
TBS	tributylsilane
TIC	total ion chromatogram
TCP	tetrachlorophthalimide
TEA	triethylamine
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl radical
TES	triethylsilane
Tf	trifluoromethanesulfonyl (or triflyl)
TFA	trifluoroacetic acid
TFAA	trifluoroacetic anhydride

THF	tetrahydrofuran
TLC	thin layer chromatography
TMC	*N*,*N*,*N*-trimethyl chitosan
TMG	*N,N,N*-trimethyl-D–glucosamine
TMS	trimethylsilane
TOF	time of flight mass spectrometer
Tol	tolyl
TPS	triphenylsilane
Troc	trichloroethoxycarbonyl
Ts	tosyl
UDP	uridine diphosphate
UMP	uridine-monophosphate
UppP	undecaprenyl pyrophosphate phosphatase
UppS	undecaprenyl phosphate synthase
Val	valine
vs	versus

Thesis Structure

The results presented in this thesis were developed during March 2015 – November 2018. This thesis is divided in three major chapters.

Chapter I – consists of a brief state of the art of the current knowledge on the biosynthesis of the bacterial peptidoglycan (PGN) and chitin.

Chapter II – covers the synthetic studies that were developed to modify glucosamine mono- and disaccharides in a chemo- and regioselective way towards NAG-NAM disaccharides and precursors of chitooligosaccharides (COSs).

This chapter describes a novel and simple synthetic scheme developed to obtain a versatile precursor of NAG-NAM oligosaccharides from a fully protected chitobiose. This process takes advantage of the chitobiose β-(1,4) glycosidic bond, which avoided the difficult enantioselective glycosylation reaction for the disaccharide assembly. Peracetylated chitobiose was used as starting material and the use of different protecting groups allowed the regioselective introduction of the critical lactyl unit of a versatile intermediate in 5 synthetic steps. This chapter also describes the synthetic studies performed to reach important intermediates by applying unconventional synthetic strategies to modify glucosamine derivatives.

Chapter III – reports the different approaches investigated to attain novel bacterial cell wall surrogates, in particular the oligosaccharides that were obtained from chitosan and that mimic the carbohydrate basic skeleton of most bacterial cell surfaces as they may be recognized by different molecular PGN recognition systems or bacterial enzymes involved in the maturation of PGN. In this chapter an innovative approach involving the chemical modification of chitosan, using a molecular clamp based strategy, was developed to produce *N*-acetylglucosamine-*N*-acetylmuramic (NAG-NAM) containing oligomers. Intercalation of NAM residues were confirmed through the analysis of oligosaccharide fragments released upon enzymatic digestion with a PGN hydrolase. This approach allowed us to determine that the developed synthetic route allowed the production of NAG-NAM containing oligosaccharides in 33% yield. This strategy combines steps of chemical modification and

enzymatic digestion and provides a novel and simple route for an easy access to bacterial cell wall fragments – biologically important targets.

General conclusions – this final chapter includes a summary of all results obtained and approaches investigated. The results obtained may serve as a conceptual framework to the design of novel synthetic strategies towards PGN oligosaccharides. In this chapter, I also propose procedures for the isolation and purification of these PGN oligosaccharides.

The results obtained in this thesis are published in two international and peer-reviewed journals:

- "A top-down chemoenzymatic approach towards N-cetylglucosamine-N-acetylmuramic oligosaccharides: chitosan as reliable template", **Fausto Queda**, Gonçalo Covas, Tomé Silva, Cátia A. Santos, Maria R. Bronze, Francisco J. Canada, Marta C. Corvo, Sérgio R. Filipe, Maria M. B. Marques, *Carbohydr. Polym.*, **2019**, accepted. DOI: 10.1016/j.carbpol.2019.115133
- "From a natural polymer to relevant NAG-NAM precursors", Luísa C. R. Carvalho, **Fausto Queda**, Cátia V. Almeida, Sérgio R. Filipe and M. Manuel B. Marques; *Asian J. Org. Chem.,* **2018**, *7*, 2544-2551.
- "Selective Modification of Chitin and Chitosan: on the route to tailored oligosaccharides", Luísa C. R. Carvalho, **Fausto Queda**, Cátia V. Almeida Santos and M. Manuel B. Marques, *Chem. Asian J.,* **2016**, *11*, 3468-3481.

And have been presented as oral/panel presentations in different national and international meetings:

***Oral presentations*:**

- "Bacterial Cell Wall Surrogates from Chitosan: a new recognition system", Fausto Queda, Gonçalo Covas, Cátia Almeida Santos, Tomé Silva, Maria R. Bronze, Francisco Javier Canada, Sérgio R. Filipe and M. Manuel B. Marques – 29th International Carbohydrate Symposium, ICS2018, Lisbon (Portugal), 14-19/07/2018.
- "Bacterial Cell Wall Surrogates from Chitosan: a new molecular recognition system", Fausto Queda, Gonçalo Covas, Cátia Almeida Santos, Tomé Silva, Maria R. Bronze, Francisco Javier Canada, Sérgio R. Filipe and M. Manuel B. Marques – 6th Portuguese Young Chemists Meeting, 6PYCHEM, Setúbal (Portugal), 15 – 18/05/2018.

Panel presentations:

- "From chitin to bacterial PGN fragments", Fausto Queda, Cátia Santos, Luísa C. R. Carvalho, M. Manuel B. Marques – 2nd International Caparica Christmas Conference on Translational Chemistry, IC3TC, 4-7/12/17.
- "An innovative approach towards bacterial cell Wall oligosaccharides", Fausto Queda, Tomé Silva, Cátia Santos, Gonçalo Covas, Maria R. Bronze, Francisco Javier Canada, Sérgio R. Filipe and M. Manuel B. Marques – XXV Encontro Nacional da SPQ, Lisboa (Portugal), 16-19/07/2017.

- “An innovative approach towards bacterial cell wall oligosaccharides”, Fausto Queda, Tomé Silva, Cátia Santos, Gonçalo Covas, Sérgio Filipe, Francisco Canada, Maria Bonze, Maria Marques, 19th European Carbohydrate Symposium, EUROCARB, Barcelona (Spain), 2-6/07/2017.
- “Studies towards modified chitosan: A new approach to NAG-NAM“, Fausto Queda, Gonçalo Covas, Sérgio R. Filipe, Maria Manuel Marques – 11th International Meeting of the Portuguese Carbohydrate Group and 6th Iberian Carbohydrate Meeting, GLUPOR11, Viseu (Portugal), 6-10/09/2015.
- “Chitosan as renewable resource for the synthesis of NAG-NAM moiety”, Fausto Queda, Gonçalo Covas, Cátia Almeida Santos, Tomé Silva, Maria R. Bronze, Francisco Javier Canada, Sérgio R. Filipe and M. Manuel B. Marques – 2nd EuChemMS Congress on Green and Sustainable Chemistry, 2nd EuGSC, Lisbon (Portugal) 4-7/10/2015.

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1 Bacteria overview

1.1 Introduction

1.1.1 Carbohydrate Polymers of β-(1,4) linked *N*-acetylglucosamine (NAG)

Carbohydrates, the most abundant class of organic compounds in nature, are essential components of the cell surface of both bacteria and mammalian cells, and are involved in different biological phenomena such as cell-cell communication and pathogen infection.[1-10] In particular, 2-amino-sugars play an important role on biological cell surfaces and are, therefore, attractive targets for medicinal chemistry and biological research. Most of these carbohydrates exist as polysaccharides, glycoconjugates or glycosides linked to other carbohydrate units *via O*-glycosidic bonds. Among the biologically relevant carbohydrates are the glycoconjugates possessing residues of 2-amino-2-deoxy-β-D-glucopyranosyl (D-glucosamine) series. The most representative examples of natural oligosaccharides containing D-glucosamine and exhibiting a relevant biological role are depicted in Figure 1.1: chitin and chitosan, the natural and abundant biopolymers that are present in the crustaceans shells, fungi and other cephalopod, and that consists of β–(1,4) linked *N*-acetylglucosamine (NAG) repeating units;[11, 12] the Nod[13] (chitooligosaccharides – COS) and the Myc factors [14](lipochitooligosaccharides), that are involved in the signalization and root nodule formation and that contain a sequence of three to four NAG units coupled to an N-substituted unit at the non-reducing end, linked by β–(1,4) glycosidic bonds between NAG units. Peptidoglycan (PGN), also known as murein, a major component of the bacterial cell wall, is made of repeating *N*-acetylglucosamine (NAG)–*N*-acetylmuramic (NAM) disaccharide units, linked via [NAG-(β-1,4)-NAM] linkage, with stem peptides attached to the D-lactyl (Lac) moiety of each NAM.[15]

Indeed, the presence of the NAG moiety is recurrent in biologically important oligosaccharides and glycoconjugates since they play a key role in a wide range of biological processes. However, the molecular details of carbohydrate-mediated recognition events are still not completely unraveled.

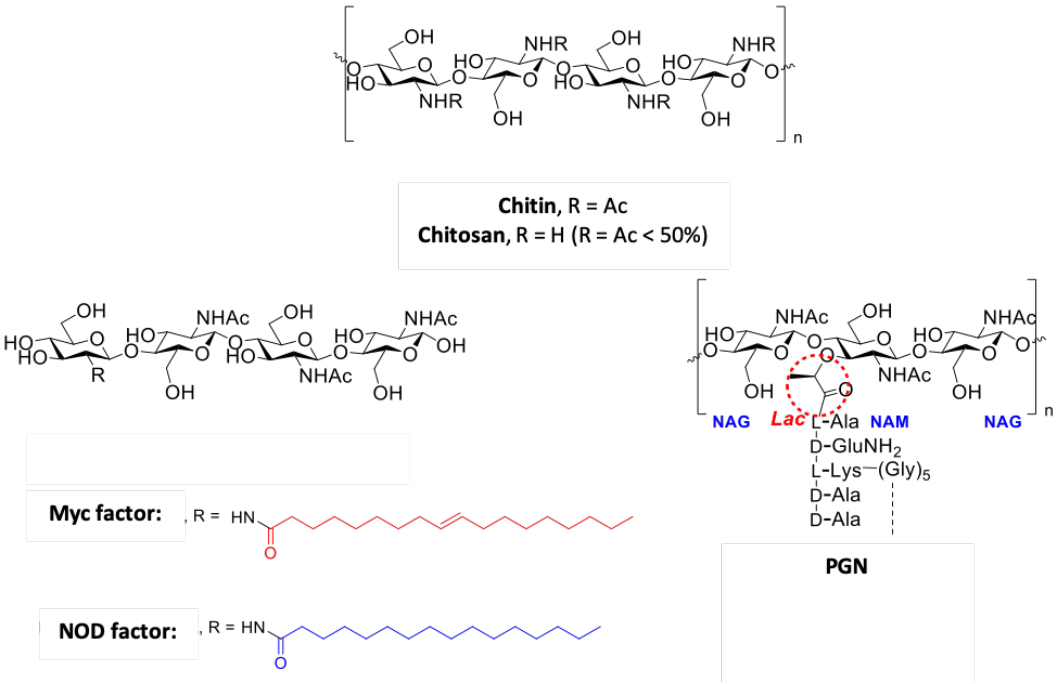

Figure 1.1 Structures of oligosaccharides containing N-acetyl-D-glucosamine (NAG)

1.1.2 Peptidoglycan biosynthesis

N-acetyl glucosamine (NAG or GlcNAc) is a major constituent of the PGN that is present in the bacterial cell wall and of chitin that is present in the fungal cell wall and of invertebrates. The activated form of this amino sugar, the UDP-GlcNAc, is frequently used by these different organisms for biosynthesis purposes. In the production of PGN, three enzymes[16] are used in four sequential steps onto UDP-GlcNAc biosynthesis, Figure 1.2. The first step is the conversion of fructose-6-phophate (Fru-6-P) into glucosamine-6-phosphate (GlcN-6-P) by glucosamine-6-phosphate synthase (GlmS).[17] Next step GlcN-6-P is converted by phosphoglucosamine mutase (GlmM) in glucosamine-1-phosphate (GlcN-1-P).[18] The two final steps of this pathway involve the transfer of an acetyl and uridyl groups leading to the formation of *N*-acetylglucosamine-1-phosphate (GlcNAc-1-P) and finally UDP-GlcNAc, respectively. These final two steps are catalyzed by the same enzyme, *N*-acetylglucosamine-1-phosphate transferase (GlmU).[19]

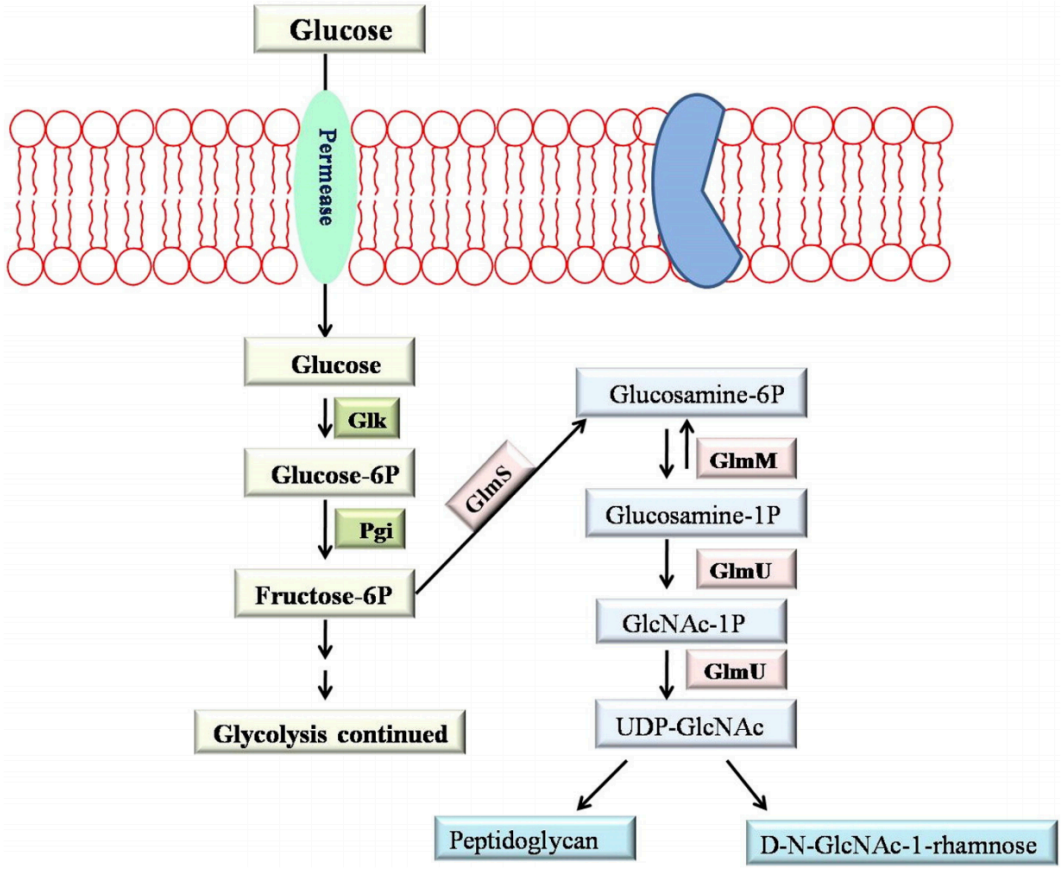

Figure 1.2 Schematic representation of UDP-N-acetylglucosamine synthesis. Glk, glucokinase; Pgi, phosphoglucoseisomerase; GlmS, glucosamine-6-phosphate synthase; GlmM, phosphoglucosamine mutase; GlmU, glucosamine-1-phosphate acetyltransferase/N-acetylglucosamine-1-phosphate uridyltransferase.[20]

The synthesis of PGN takes place in three separated bacterial compartments: in the cytoplasm, at the membrane and in periplasmic/external space. It starts with the previously described UDP-GlcNAc synthesis in the cytoplasm site, Figure 1.3. Then the UDP-N-acetylmuramyl pentapeptide (UDP-Mpp) is synthesized, using as UDP-GlcNAc[21, 22] as the initial substrate, by the addition of different aminoacids that is carried out by the action of Mur enzymes (MurA, MurB, MurC, MurD, MurE and MurF). The final cytoplasmic PGN precursor is then embedded into the membrane and linked to undecaprenyl phosphate molecules whose synthesis is assisted by the undecaprenyl phosphate synthase (UppS). This enzyme catalyzes successive condensation reactions of farnesyl pyrophosphate (FPP) and eight isopentenyl pyrophosphates (IPP) to produce the molecule of undecaprenyl pyrodiphosphate (C55-PP). Then a dephosphorylation takes place by the undecaprenyl pyrophosphate phosphatase (UppP) to produce undecaprenyl pyrophosphate (C55-P).[23, 24]

The first membrane reaction step of PGN synthesis is catalyzed by the phospho-MurNAc-pentapetide translocase (MraY) that uses the two substrates of UDP-Mpp and C55-P.

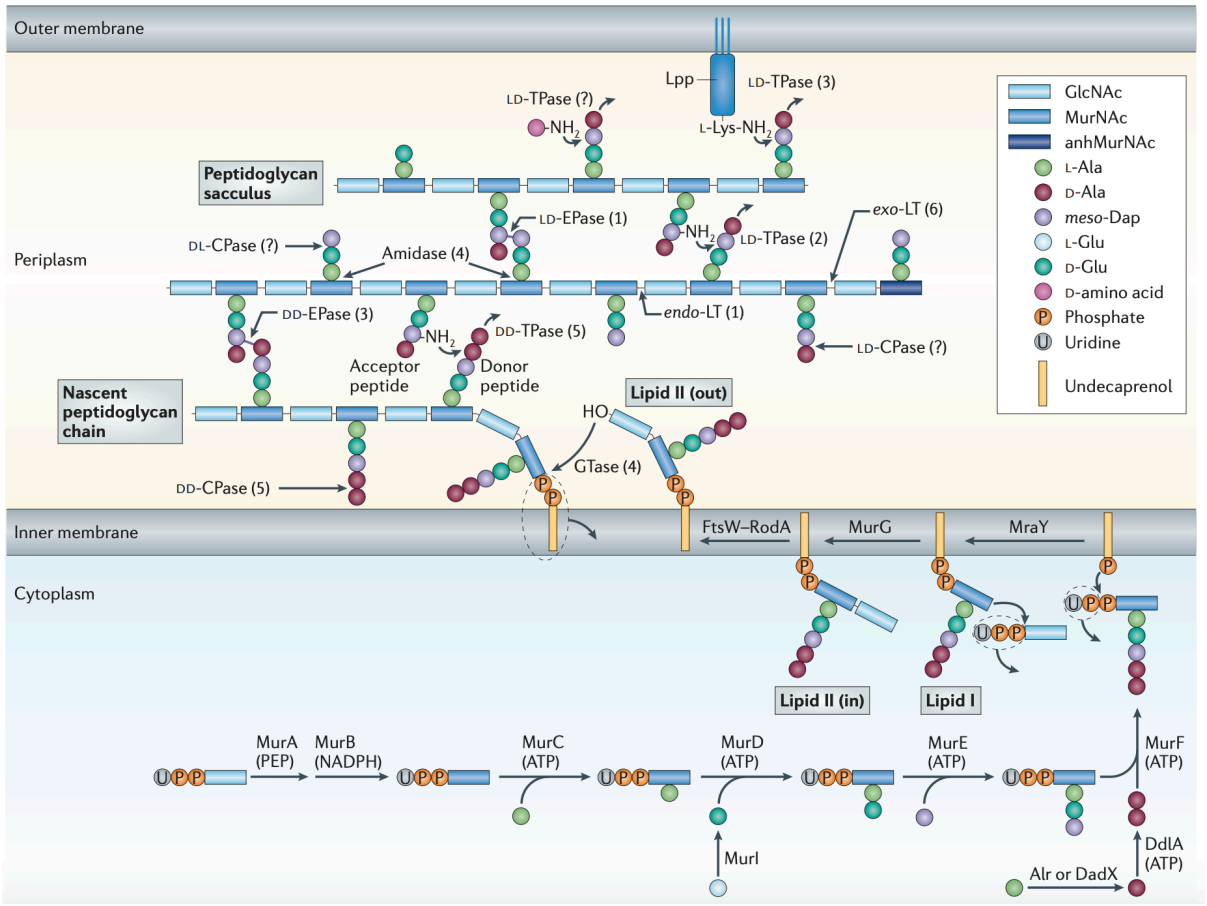

Figure 1.3 The synthesis and attachment of a new peptidoglycan stand to the existing.[25]

Phospho-MurNAc-pentapetide moiety is transferred from UDP-Mpp to C55-P forming uridine-monophosphate (UMP) and undecaprenyl-pyrophophoryl-MurNAc-pentapetide, also known as Lipid I.[26, 27]

Then a GlcNAc moiety from a soluble UDP-GlcNAc precursor is transfered to Lipid I by the glycosyltransferase MurG to produce Lipid II. Consequently, Lipid II is transported from the cytoplasm to the outer membrane space by the action of the Lipid II flippase. This step was originally thought to be carried by the FtsW/RodA enzime[28] but more recently growing evidence have been produce to associate this activity to the MurJ like enzymes.[29]

In the final steps of PGN synthesis, glycosyltransferases (GTases) are required to polymerize the glycans chains and DD-transpeptidases (DD-TPases), or L,D-transpeptidases, to crosslink the peptides present in neighboring glycan chains.[24] Bacteria produce different penicillin-binding proteins (PBPs) (also called TPases), which are involved in the synthesis of the

bacterial PGN, an that can be divided in three types: the bifunctional GTase-TPases (A PBPs), the monofunctional TPases (B PBPs) and the monofunctional GTases.[24] Three bifunctional synthases (PBP1A, PBP1B, and PBP1C), a GTase (MgtA) and two TPases (needed for cell elongation (PBP2) or cell division (PBP3)) are found in *E. coli*.[30] PBP1B is responsible for glycan polymerization, 28 disaccharide units, and peptide crosslinking 40-50% of peptides. PBP1A generates shorter glycan strands (20 disaccharide units) and 22% of peptide crosslinking.[31] Through labeling experiments, it was confirmed that PGN grows by insertion of fresh synthesized glycans, in the existing PGN chains.[32, 33] The fact that no oligomers intermediates were found in the cell suggests the polymerization and transpeptidation steps occur at the same time, which supports the existence of bifunctional enzymes.[34]

1.1.3 Bacterial cell wall

Bacteria can be divided in two major groups according to the composition and organization of their bacterial cell surface: the Gram-positive bacteria, frequently producing a lysine-type PGN, and the Gram-negative bacteria, usually with a DAP-type PGN, as it is shown in Figure 1.4. The bacteria cell wall present in Gram-positive bacteria has in its composition a cytoplasmatic lipid membrane, the macromolecule of PGN, also called murein, which may be covered by teichoic acids (TA) or other polysaccharides. The bacterial cell surface of Gram-negative bacteria has in its composition a cytoplasmatic membrane, a thin layer of PGN, which is concealed by an outer membrane that carries porins and lipoproteins. Different polysaccharides, such as the LPS (also termed as endotoxin), are also linked to the outer membrane.

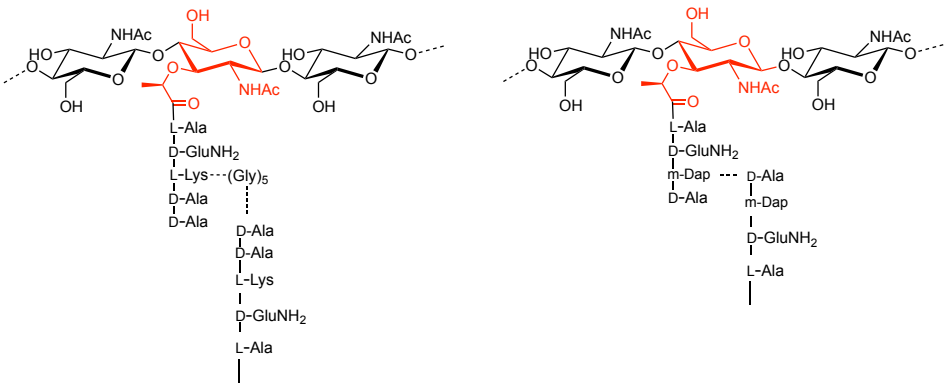

Figure 1.4 Bacteria PGN atomic/skeleton structure representation highlighting the NAM unit. The repeating unit of the PGN produce by *S. aureus* (Gram-positive bacteria) (Left) and *E. coli* (Gram negative bacteria) (Right) are shown.

The organization of bacterial cell wall frequently explains the different characteristics and properties, such as thickness and permeability of the cell-wall, associated with these two groups of bacteria, Figure 1.5.

PGN is a major component of the bacteria cell wall, is normally composed by a disaccharide, monomer, of NAG linked via β–(1,4) glycosidic bond to a NAM, which is connected to a penta-peptide that may include aminoacids such as L-Ala, D-iso-Gln, L-Lys, D-Ala (frequently present in Gram-positive bacteria) or L-Ala, D- *iso*-Glu, m-DAP, D-Ala (observed in several Gram-negative bacteria). Despite this minimal chemical composition, several modifications in both the glycan structure, and the aminoacids included in the cross-linking bridge and the peptide stem, have been reported leading to numerous new structures of PGN. These modifications can be as varied as a glycine in the first position, a D-*iso*-glutamate in the second position or a D-serine in the fifth position.[35]

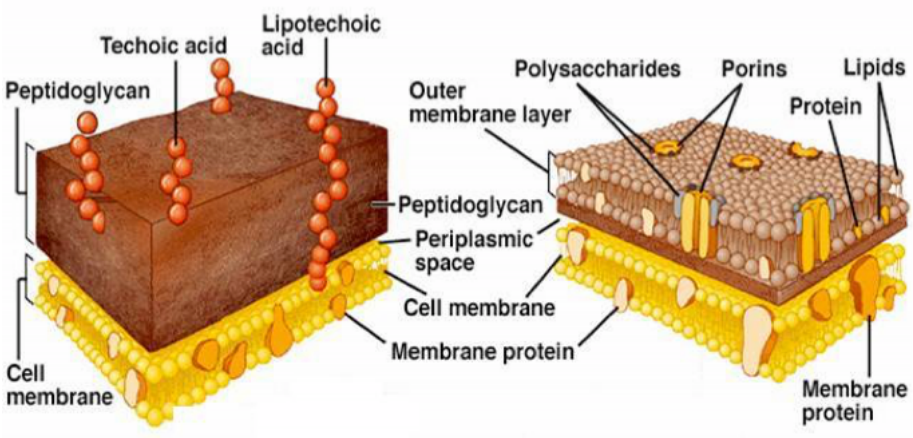

Figure 1.5 Bacteria cell-wall representation. Gram-negative and Gram-positive respectively.[26]

1.1.3.1 Modifications on PGN carbohydrate backbone

Some bacteria have developed strategies to protect their own PGN from the action of PGN hydrolases present in the growth medium, such as the host lysozyme-like enzymes. Chemical modifications of the NAG and NAM residues may help bacteria to evade the host immune system. Figure 1.6 summarizes the major changes in the PGN sugar backbone that may protect bacteria from the host immune system.[36]

The *O*-acetylation in NAM units in Gram-negative bacteria gives lysozyme resistance and resistance to autolysis (lysis induced by the activity of the PGN hydrolases produced by the bacteria).[37] In the case of Gram-positive bacteria, the same modification gives lysozyme

resistance, resistance to macrophages killing and penicillin resistance.[38, 39] Contrary to NAM, O-acetylation in NAG units is not frequently observed in the PGN of bacteria. Although it seems to give no resistance to the activity of lysozyme, it provides resistance to the action of *N*-acetyl-glucosaminidase Acm2, an enzyme produced by *L. plantarum*.[40]

The N-deacetylation of NAG units also provides resistance to the activity of lysozyme and to neutrophil killing.[41] When present in the NAM unit, N-deacetylation helps bacteria to evade the immune system since the NAc group from the NAM unit seems to be required in the recognition of this PGN fragment by NOD2.[42]

In certain bacteria, such as *Mycobacterium tuberculosis*, PGN may be modified by reaction of N-glycolylation in NAM units, which gives bacteria the ability to resist to lysozyme, β-lactam resistance and promotes the cytokine response.[43, 44]

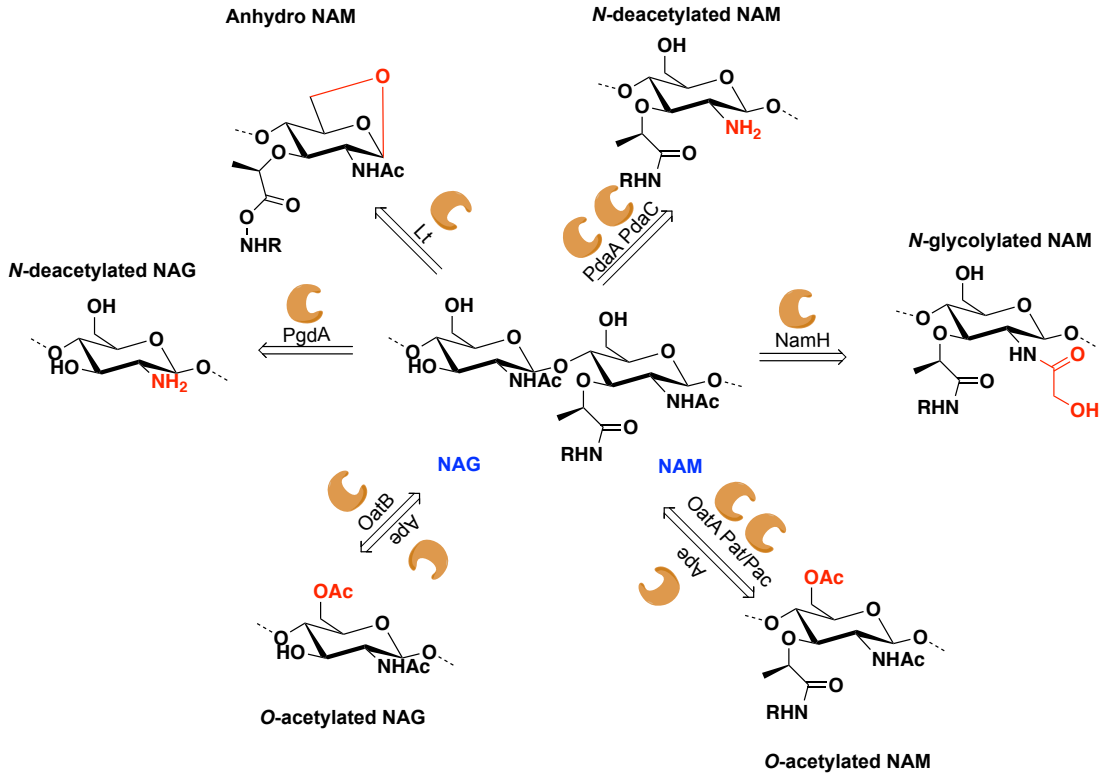

Figure 1.6 Schematic representation of various modifications among PGN sugar backbone, highlighting the structural modification.[45]

1.1.4 Chitin biosynthesis

Chitin, one of the most abundant polymers in nature and the most abundant amino polymer, is synthesized by fungi, nematodes and arthropods. Chitin is a poly–β(1,4)–NAG polymer that has the role of maintaining the strength of the cell wall of fungi (it can be found in 1-15% of the cell wall mass) and the exoskeletons of other organisms. Chitin biosynthesis carried out

by membrane-bound chitin synthases. UDP-GlcNAc is used as substrate by these enzymes to form poly–β(1,4)–NAG. Chitin is first elongated by adding GlcNAc to the nonreducing end of the already present polymer and then extruded to the cell wall space by the reducing end. The polymeric chitin present within the cell wall matrix can be assembled into microfibrils, through interchain hydrogen bonding, and arranged in antiparallel pattern.[46]

Different enzymes are involved in the overall synthesis of chitin. In Figure 1.7, Glycogen phosphorylase (1) converts glycogen (a polymer of glucose residues that is used as a form of energy source) in Glc-1-P, which can be then used by the enzymes involved in glycolysis or trehalose synthesis after the action of a phosphoglucomutase (4). On the other hand, glucose residues are converted into fructose-6-P by the action of hexokinase (3), phosphoglucomutase (4) and glucose-6-P isomerase (5). The chitin pathway synthesis begins with glutamine-fructose-6-phostate amindotransferase (6, GFAT) and fructose-6-P. The transference of ammonia from 1-glutamine to fructose-6-P and then isomerization form fructosamine-6-P. After, an acetyl group from coenzyme A is transferred to fructosamine-6-P by gluctosamine-6-P acetyltransferase (7) to afford GlcNAc-6-P. By the action of phosphoacetylglucosamine mutase (8) the phosphate group is transferred from the C6 to C1. By the action of UDP-GlcNAc pyrophosphorylase (9) the uridiyl group transferred to obtain UDP-GlcNAc which is the substrate to the chitin synthase (10). Assembled chitin polymers can be hydrolyzed by chitinases (11) and N-acetylglucosamidases (12) to generate GlcNAc, which can be reused to a new chitin biosynthesis, Figure 1.7.[47]

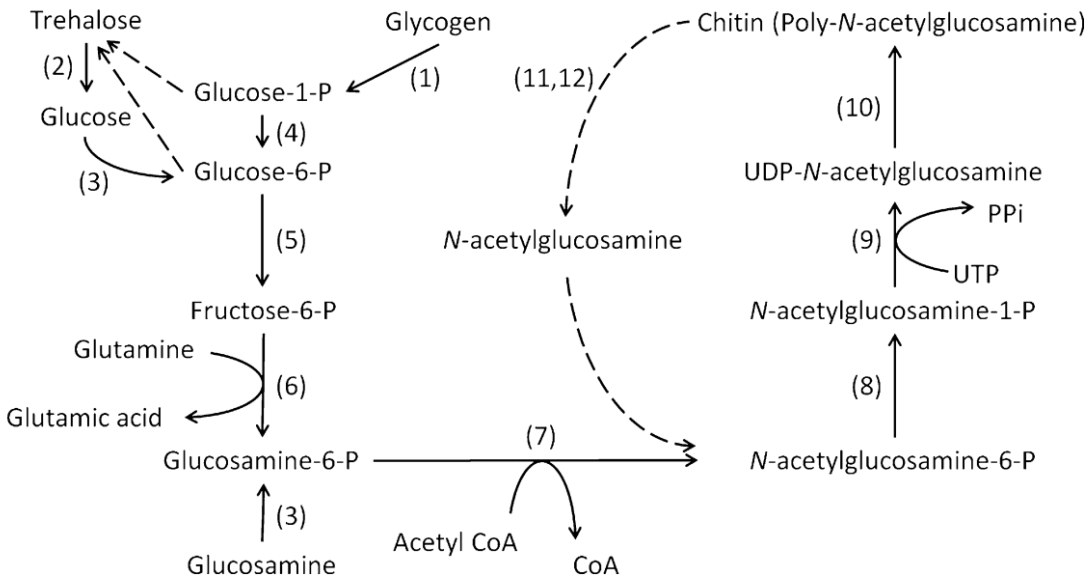

Figure 1.7 Chitin synthesis pathway in fungi and insects.[47]

The selective construction of NAG glycosides has been a significant task in carbohydrate chemistry, and despite the synthetic advances remains a major challenge.[48-52] Due to the remarkable biological role of the NAG-containing oligosaccharides, special efforts have been dedicated to search for efficient synthetic approaches to such complex molecules, involving efficient, simple, stereo- and region- selective methods.[53-55] One of the most efficient strategies to prepare oligosaccharides consists in the preparation of key building blocks of di-, tri, and higher-oligosaccharides, that can be applied to assemble complex molecules.[50, 53-58] Thus, a critical limitation for further PGN investigation, is the limited availability of PGN in pure form from natural sources, and the need of ligand purification in reliable amounts. Indeed, the extraction and purification process of PGN presents several difficulties, due to the presence of TA and proteins that can contaminate the PGN fragments.[59-62]

In this chapter both NOD and Myc factors were not covered since the main focus is PGN and chitin, synthesis and modifications.

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2 Synthetic studies towards a NAG-NAM disaccharide

2.1 Introduction

2.1.1 Glucosamine -a challenging scaffold

The most important classes of natural oligosaccharides and glycoconjugates contain residues of 2-amino-2-deoxy-β-D-glucopyranosyl (D-glucosamino) moieties. D-Glucosamines exist in several biological systems, such as the cell membrane of living organisms, acting as bacterial receptors or signaling molecules, as antibody recognition sites, as tumor markers, or as hormonal and enzymatic glycoproteins.[1] Glucosamines are also part of important structural oligosaccharides.

One of the most critical limitations in investigating the biological functions of these complex glycostructures is their limited availability and purity from natural sources. Consequently, there has been an increasing demand towards synthetic approaches that provide pure material of these oligosaccharides and glycoconjugates, or modified structures, for biological investigations.

The majority of naturally abundant glycoconjugates contain N-acetylated D-glucosamino moiety that are connected through a 1,2-*trans* linkage. Thus, it would be desirable to use the commercially available *N*-acetyl D-glucosamine for syntheses towards these glycoconjugates. However, the N-acetyl group poses several limitations to the glycosylation reaction with respect to both donor and acceptor units.

Indeed, the glycosylation of complex aglycones with glycosyl donors bearing a 2-acetamido-2-deoxy functionality is usually impractical due to the formation of a 1,2-O,N-oxazoline intermediate during glycosylation, which is a stable intermediate, Figure 2.1 A, and drastically decreases the rate and yield of the glycosylation step, especially when the chosen glycosyl acceptors have poor nucleophilicity or are sterically hindered. Also, concerning 2-acetamido-2-deoxy functionality as acceptor the amide group can establish a hydrogen bond with the hydroxy group O-4 decreasing the nucleophilicity at this position, Figure 2.1 C.

In order to avoid the formation of the stable 1,2-O,N oxazoline intermediate and improve the formation of 1,2-*trans* glycoside several disubstituted 2-amino2-deoxyglycosyl donors have been developed, Figure 2.1 B. The use of these protecting groups result on the blocking of the amino group either by using two different protecting groups or a bivalent protecting group.

Therefore, donor activation results in the formation of an unstable oxazolinium intermediate that blocks the α-face.[2]

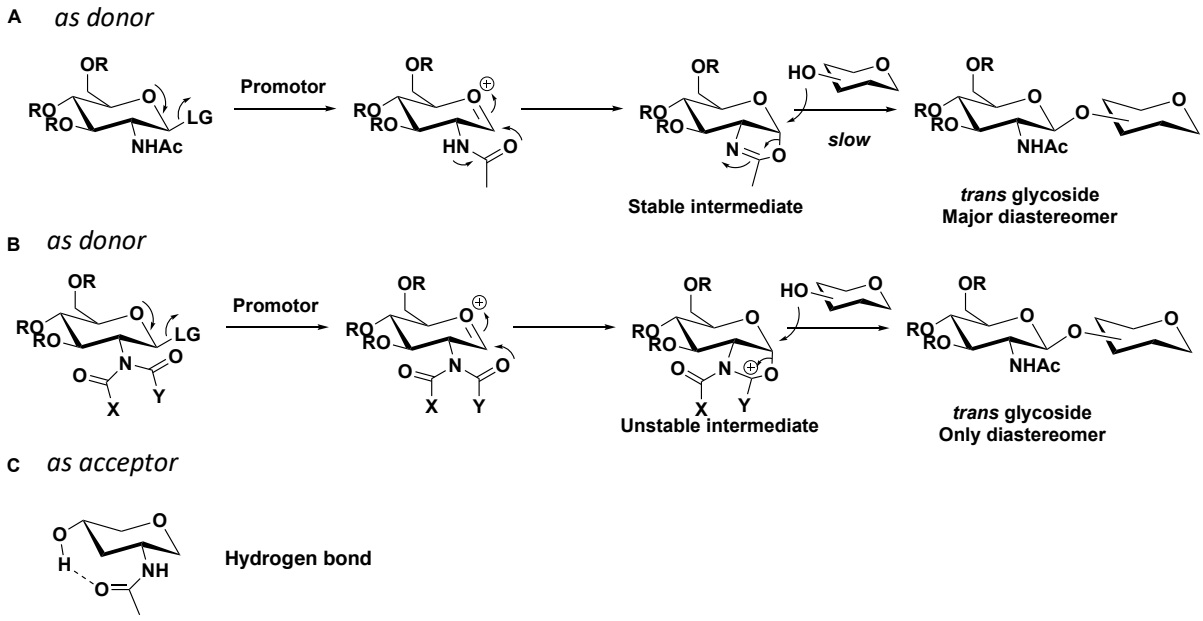


Figure 2.1 Influence of the C-2 group on the glycosylation stereoselectivity.(adapted from[2])

To overcome the problems associated with the use of NAG, Figure 2.1, many protecting groups have been employed. Figure 2.2 depicts some of the most used N-protecting groups applied in glucosamine derivatives.

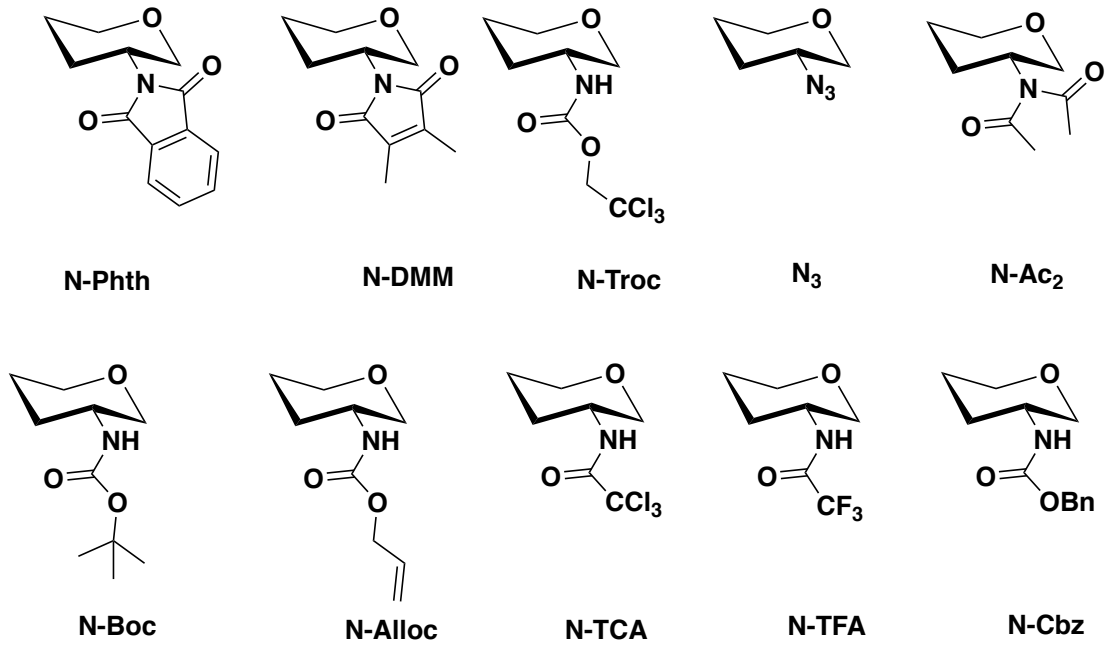
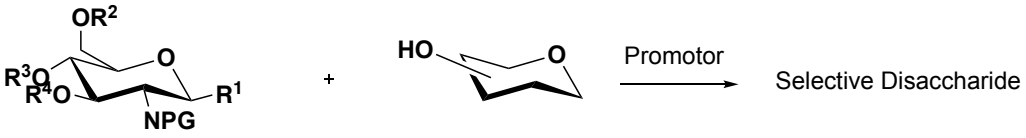
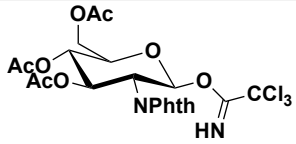
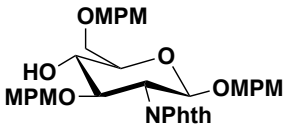
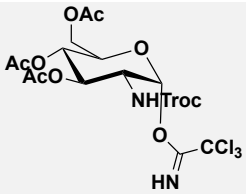
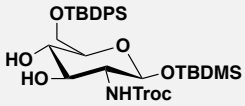
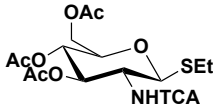
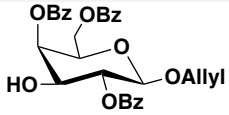
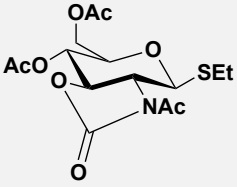
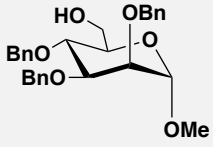
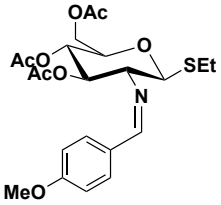
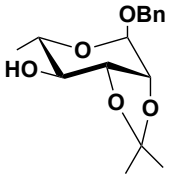


Figure 2.2 Most common amino protecting groups.

Bivalent protecting groups block the stability of the 1,2-O,N-oxazoline however, usually turn the glucosamine moiety more bulky. Monovalent protecting groups can provide well define selectivity towards the glycosylation reaction and also improve the solubility in organic solvents, comparing with *N*-acetyl D-glucosamine. The choice of the protecting group must be planned to take into consideration the synthetic steps involved, and reaction conditions used. Thus, most synthetic plans designed to afford glucosamine conjugated or oligosaccharides rely on the use of properly protected glucosamine moieties, in which the N-protecting group is modified during the synthetic sequence.[3]

Table 2.1 Different glycosylation conditions and their selectivity.



Entry	Donor	Acceptor	Promotor	Solvent	T (ºC)	Yield (%)	Selectivity (α:β)
1[4]			TMSOTf	DCM	-20	91	β
2[5]			TMSOTf	DCM	0	76	β
3[6]			NIS, TfOH	DCM	- 20	86	β
4[7]			DMTST, DTBMP	DCM	0 - 20	94	β
5[8, 9]			Ni(4-F-PhCN)$_4$(OTf)$_2$	DCM	25	93	12:1

The 2-amino protecting groups play a key role in glycosydic bond formation involving glucosamine derivatives acting both as donors and acceptors. Accordingly, several N-protecting groups have been developed not only with regard to their compatibility with diverse carbohydrate synthetic methods but also to their ability to direct the stereochemical outcome of the glycosylation reaction. Table 2.1, summarizes some examples on the importance of the N-protecting group regarding the desired selectivity of the new glycosylic bond.

2.1.2 Synthetic routes towards peptidoglycan fragments

The synthesis and composition of the PGN is associated with expression of bacterial resistance to different antibiotics and with a variety of host/bacteria interactions. The determination of the role of PGN in host disease has been hampered by the lack of pure and homogeneous polymerized PGN.[10] The major limitation encountered in these studies is the limited availability of pure PGN fragments obtained through purification procedures from natural sources. Thus, there is a need to have pure and well-defined PGN fragments in order to have viable biological studies. To circumvent this bottleneck, we and others have been developing strategies for the synthesis of muropeptides from glucosamine residues.

In 2001, Fukase and his co-workers reported the synthesis of PGN fragments.[11] On the follow-up of their previous report from the on the synthesis of partial structures of cell wall PGN,[12] the authors developed an orthogonal synthesis in order to create building blocks to form the complex octasaccharide $(NAG\text{-}NAM)_4$ (**II.9**) with two amino acid strands linked to every NAM unit, Figure 2.3.

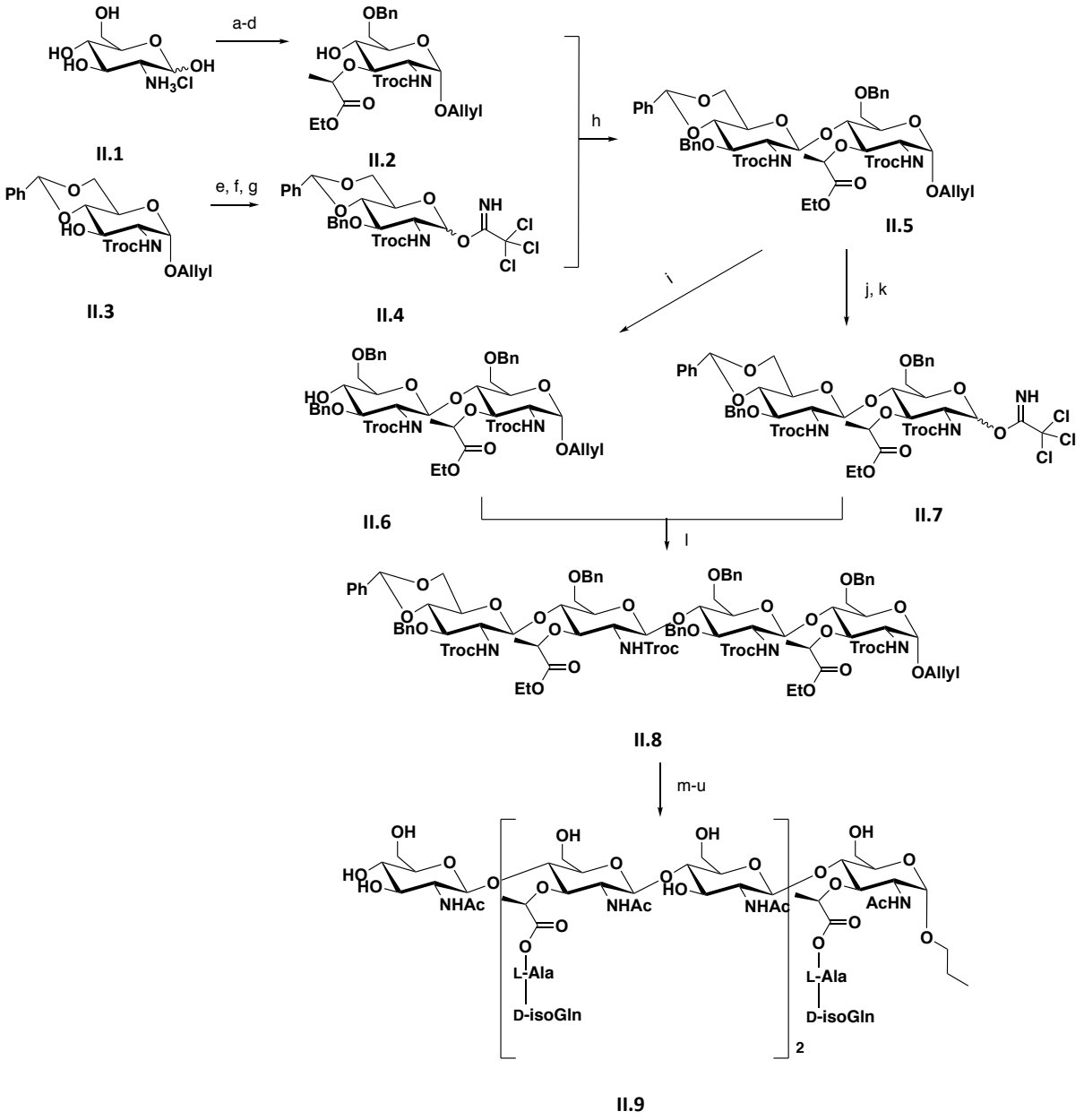


Figure 2.3 Synthesis of PGN fragment II.9 reported by Fukase[11]; (a) i) AllocCl, $NaHCO_3$, H_2O; ii) AllylOH, DOWEX 50 W X8 200–400 mesh, 80 °C; iii) $PhCH(OMe)_2$, *p*-TsOH, 57%; (b) NaH then trifluoromethanesulfonyl-L-(*S*)-2-propionic acid benzyl ester, 98%; (c) $Pd(PPh_3)_4$, AcOH, DCM, then TrocCl, 58%; (d) $Me_3N{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, ACN, 87%; (e) BnBr, AgO, DCM, 87% (f) Ir complex, H_2, THF, then I_2, H_2O, 99%; (g) CCl_3CN, Cs_2CO_3, DCM, quantitative; (h) TMSOTf (0.1 equiv.), MS 4 Å, DCM, −15°C, 88%; (i) $Me_3N{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, ACN, 52%; (j) Ir complex, H_2, THF, then I_2, H_2O, 91%; (k) CCl_3CN, Cs_2CO_3, DCM, 85%; (l) TMSOTf (0.1 equiv.), MS 4 Å , DCM, −15 °C, 79%; (m) $Me_3N{\cdot}BH_3$, $BF_3{\cdot}Et_2O$, ACN, 60%; (n) Ir complex, H_2, THF, then I_2, H_2O, 81%; (o) CCl_3CN, Cs_2CO_3, DCM, 83%; (p) TMSOTf (0.1 equiv.), MS 4 Å , DCM, −15 °C, 70%; (q) Zn–Cu, AcOH, then Ac_2O, Py, 82%; (r) 1 M NaOMe, THF, H_2O: LiOH, dioxane, THF, H_2O, 97%; (s) HCl·H-L-Ala-D-Glu(OBn)-NH_2, WSCI·HCl, HOBt, TEA, DCM, 31%; (t) $Pd(OH)_2$, AcOH, H_2 (10 atm), 48%.

The first step of this synthesis consisted in the protection of the amine group of D-glucosamine with the allyloxycarbonyl (Alloc) group, by treatment of the glucosamine with AllocCl in the presence of $NaHCO_3$ as base.

In the next step the anomeric position was protected with the O-allyl group, by reaction with allyl alcohol under acidic conditions using DOWEX, as proton exchange resin, at high temperature. The O-4 and O-6 positions were regioselectively protected via acetylidene formation by treatment with benzaldehyde dimethyl acetal catalysed by *p*-TsOH affording **II.3** in 57% yield. Nevertheless, is a well kwon procedure to regioselective protect both O-6 and O-4 positions.

The protection with the Alloc group is crucial to install the lactyl moiety at the O-3. However, this group would not provide the correct β-(1,4) selectivity during the glycosylation reaction. Nevertheless, the Alloc group is stable under the basic conditions, needed to insert the lactyl moiety. In order to obtain a β-(1,4) linkage, the authors replaced the Alloc group by the trichloroethoxycarbonyl (Troc) group. To remove the Alloc group a complex of $Pd(PPh_3)_4$, assisted by AcOH was used. The palladium complex coordinates with the Alloc double bond followed by hydrolysis with AcOH, then the TrocCl was added to provide the Troc protected product in 58% yield.[13] The use of Troc as N-protecting group on the acceptor unit did not provide any advantage but the simplification of the protecting groups removal in a later stage of the synthesis. The next step consisted on the introduction of the lactyl moiety at the O-3 position. Thus, the authors used trifluoromethanesulfonyl-L-(*S*)-2-propionic acid benzyl ester after hydroxy group deprotonation using NaH, through a S_N2 reaction, in 98% yield.

Consequently, selective ring opening to provide the O-4 free ready for the glycosylation reaction (**II.2**) was performed, using $Me_3N{\cdot}BH_3$, to reduce the benzylic position, leaving the O-4 position free.

To prepare the donor unit the authors protected the O-3 position with Bn group, using BnBr, through a S_N2 reaction, catalysed by AgO, and obtained the product in 87% yield. The next step consisted on the conversion of the OAllyl group into a trichloroacetimidate group. Thus, the authors removed the O-Allyl group with an Ir complex to isomerize the double bond, promoting the H_2O nucleophilic attack at the anomeric position. The next step consisted in the treatment of the anomeric position of the donor with CCl_3CN and Cs_2CO_3 as base affording the product **II.4** in a quantitative yield.

Next step consisted on the glycosylation reaction using **II.2** as acceptor and **II.4** as donor. Since the trichloroacetimidate is an excellent leaving group, TMSOTf was used in catalytic amount, and molecular sieves, to promote the glycosylation reaction affording **II.5**, in 88% yield.

Once the disaccharide **II.5** was obtained the same rational was used to produce the tetrasaccharide **II.8**. Selective ring opening to provide the acceptor **II.6**, replacement of the O-Allyl group with trichloroacetimidate to provide the donor **II.7**. After achieving the octasaccharide the authors attached the dipeptide via solid-phase synthesis, and finally hydrogenation reaction to remove the remaining benzyl groups.

To achieve compound **II.9**, the authors carried 25 steps and an overall yield of 0.3%. This study[11] was a milestone on the synthesis of PGN fragments. To perform this synthetic plan, the authors had to carefully choose all the protecting groups, especially the amino protecting group and the anomeric position group. The N-Alloc group is a well-known group that allows many chemical modifications however, in glycosylation reactions it does not favor the β-(1,4) glycosidic bond formation. In order to overcome the N-Troc had to be installed. Also, the trichloroacetimidate group is a β-(1,4) director on glycosylation reaction.

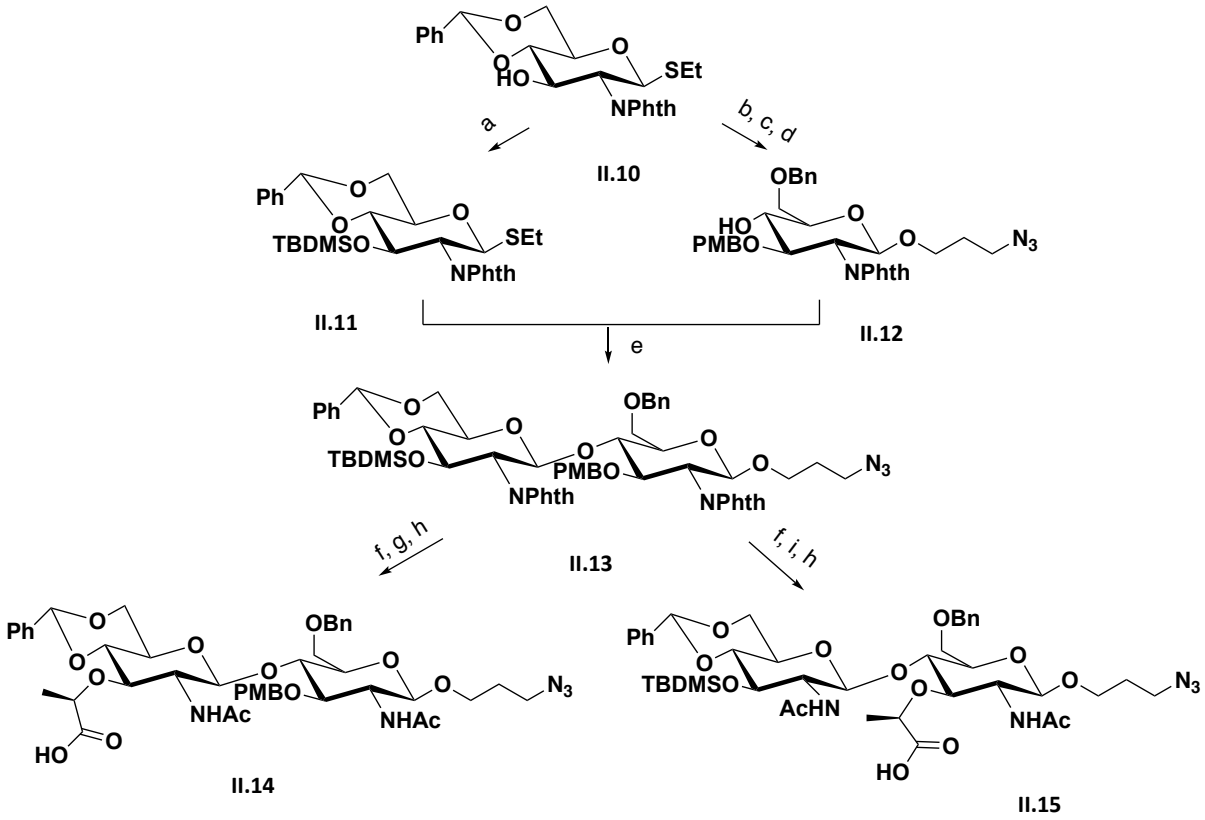


Figure 2.4 Synthesis summary to accomplish II.14 an II.15 reported by Boons[14].(a) TBDMSOTf, 2,6-lutidine, DCM, 96%; (b) PMBCl, NaH, TBAI, THF, reflux, 84%; (c) $Me_3N{\cdot}BH_3$, $AlCl_3$, THF, 72%; (d) $HO(CH_2)_3N_3$, NIS, TMSOTf, 67%; (e) NIS, TMSOTf, 62%; (f) $H_2N(CH_2)_2NH_2$, EtOH then Ac_2O, MeOH, 70%; (g) Bu_4NF, THF, 75%; (h) NaH, (*S*)-2-chloropropionic acid, dioxane, 76% to II.14 66% to II.15; (i) DDQ, DCM, H_2O, 79%.

In 2002 Boons' group reported the synthesis of a disaccharide intermediate which can be converted in NAG-NAM (**II.15**) or a NAM-NAG precursor (**II.14**), Figure 2.4.[14]

This synthetic plan describes the synthesis of a high value disaccharide intermediate. The authors started this synthesis with compound **II.10**. This compound had already the phthalimide (Phth) group as N-protecting group, the 4,6-O arylidene ring and the SEt group at the anomeric position. To prepare the donor unit the authors protected the O-3 position with TBDMSOTf, affording **II.11** in 96% yield. The conventional silylation procedure did not work and the starting material was recovered. To obtain the acceptor the authors started by protecting the O-3 position with the PMB group using PMBCl and NaH, and obtained the product in 84% yield. The next step consisted on the arylidene ring opening to give the free O-4 position using $Me_3N.BH_3$ to reduce the benzylic position, and the desired product was obtained in 72% yield. Next, the authors replaced the SEt group with 3-azido-propanol. Treatment with NIS oxidizes SEt to S(O)Et which is attacked by 3-azido-propanol to afford **II.12**, in 67% yield. The authors claimed that due to the low reactivity of O-4, self-condensation was not observed.

Having both acceptor **II.12** and donor **II.11** in hands, the next step consisted on the glycosylation reaction. The anomeric group activation with NIS promoted the nucleophilic attack by the hydroxy group of the acceptor **II.12** to afford the product **II.13** in 62%. Since the disaccharide was obtained the Phth group was no longer needed, so the authors removed it using hydrazine at reflux followed by an acetylation step, affording the acetylated product in 70% yield. Then the synthesis was divided to isolate either the NAM-NAG precursor **II.14** or the NAG-NAM precursor **II.15**. To afford **II.14** the authors removed the TBDMS group using a tetrabutylamoniun fluoride (TBAF) with a yield of 75%, followed by the introduction of the lacyl moiety. Thus, the authors used (*S*)-2-chloropropionic acid after deprotonation of O-3 with NaH, afforded the corresponding product in 76% yield. To obtain **II.15** the authors removed the PMB group using DDQ with a yield of 79%, followed by the introduction of the lactyl moiety, using the same procedure as to produce **II.14**, with a yield of 66%.

After removal of these protecting groups the disaccharides NAG-NAM and NAM-NAG were obtained. The authors picked as protecting group for the amino moiety the Phth group since it blocks the nitrogen atom, and it is only removed under hydrazide reduction. Also, the use of a thioglycoside as glycosyl donor forthe glycosylation reaction is versatile since this kind of groups are really very stable and can be activated using NIS to become a good leaving group.

The authors synthesized both disaccharides in 8 steps with an overall yield of 23,7% for the **II.14** and 9% for the **II.15**.

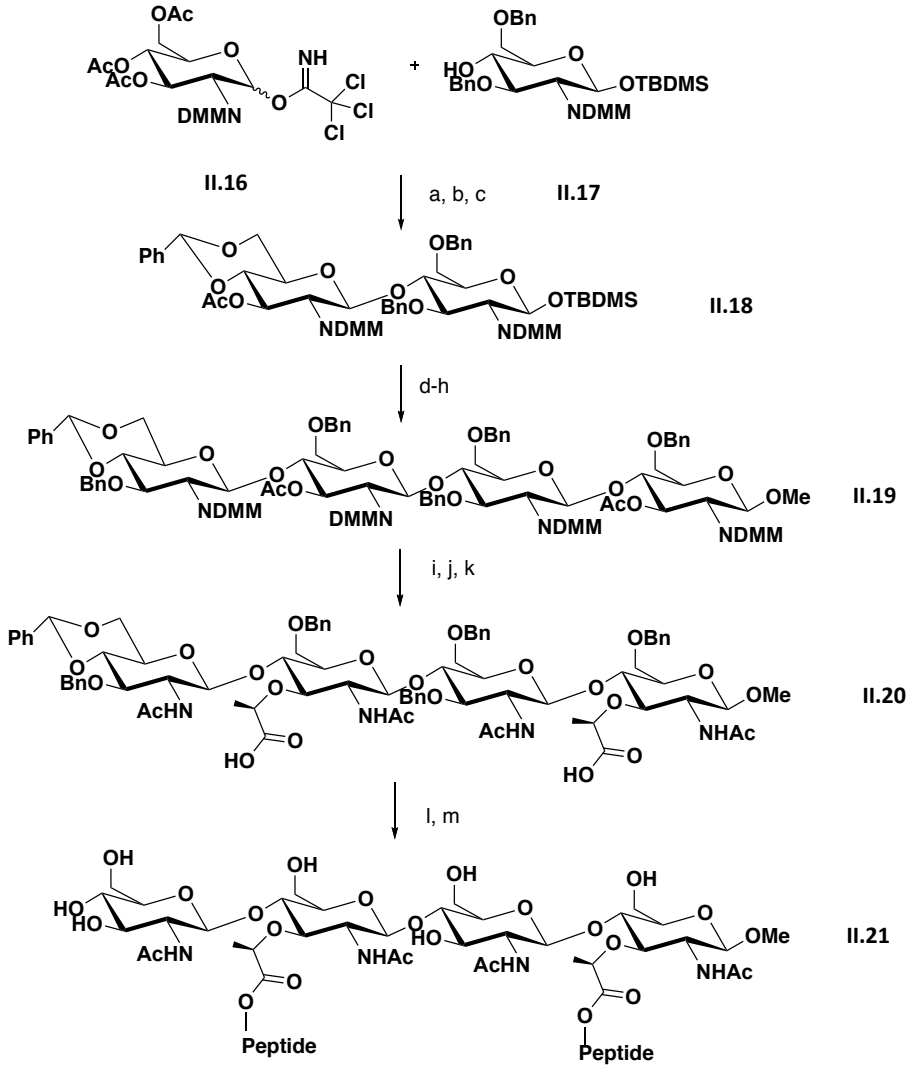


Figure 2.5 Synthesis summary to accomplish II.21 reported by Mobashery.[15] (a) TfOH, 71%. (b) (i) NaOMe, Amberlite IR-120 (H^+), (ii) benzaldehyde dimethylacetal, *p*-TsOH, 60% in two steps; (c) Ac_2O, 85%; (d) *n*-Bu_4NF, 75%; (e) Cl_3CCN, DBU; (f) monosaccharide acceptor, TfOH, 55% in two steps; (g) $BH_3.NMe_3$, $BF_3.OEt_2$, 62%; (h) monosaccharide donnor TfOH, 68%; (i) (1) NaOH, dioxane/water (4:1); HCl, pH 3.0; (2) Ac_2O, 48% in two steps; (j) NaOMe, 74%; (k) NaH, (S)-2-chloropropionic acid, 46%; (m) 4-nitrophenyl trifluoroacetate, pyridine; peptide, 41%; (l) 60% AcOH; H_2, Pd/C, 76%.

In 2004 Mobashery and his co-workers reported the synthesis of a tetrasaccharide, with two pentapeptides attached to the NAM units (**II.21**), Figure 2.5.[15]

The authors started the synthesis by preparing both **II.16** and **II.17** from glucosamine via an orthogonal synthesis. To prepare **II.16** the authors started by protecting the amino group with the dimethyl maleimide (DMM) group at the amino group, using 2,3-dimethylmaleic anhydride followed by acetylation of the hydroxy groups affording the precursor in 55% yield after the two steps. Then the authors selectively deprotected the anomeric acetyl group using

hydrazine acetate to give the free OH group at the anomeric position in 68% yield. Then the trichloroacetimidate group was introduced by treatment with CCl_3CN and DBU as base affording **II.16** in 85% yield. To prepare the acceptor **II.17**, the authors protected the hydroxy group at the anomeric position using TBDMSCl and imidazole, and obtained the sylilated product in 78% yield. Next, the acetyl groups were removed using NaOMe in methanol and the product was obtained in 73% yield. The benzylation of the O- 3 and O-6 was performed using Bu_2SnO and BnBr, **II.17** was isolated in 73% yield.

Having both acceptor **II.17** and donor **II.16** in hands, the glycosylation reaction was performed. This step was carried using TfOH as promotor affording the disaccharide in 75% yield. Three additional steps were needed to afford disaccharide **II.18**: first, removal of acetyl group using NaOMe quenched by the use of a proton exchange resin (Amberlite IR-120), and subsequent reaction with benzaldehyde dimethylacetal, leading to the formation of the 4,6-O arylidene, and then O-6 and O-3 benzylation, (60%, two steps). The last step consisted in the acetylation of the remaining O-3 free position with acetic anhydride, affording **II.18** in 85%. With this disaccharide in hands the authors needed to elongate the chain in order to create a more complex structure. Thus, the TBDMS group was removed with TBAF in 75% yield. The authors observed that for long reaction times, the yield decreased due to hydrolysis of the acetyl group. Then the trichloroacetimidate group was introduced, using the same procedure as before, followed by a glycosylation reaction with an acceptor possessing both O-6 and O-3 benzylated in 55%. Next, the selective opening of the 4,6-O arylidene with $Me_3N.BH_3$ gave the O-4 free position in 62% yield.

Then another glycosylation reaction was performed, using this trisaccharide as acceptor and a monosaccharide with 4,6-O arylidene, O-3 protected with a benzyl group and the trichloroacetimidate group at the anomeric position. The glycosylation reaction was once again catalysed by TfOH affording **II.19** in 68%.

Since the tetrasaccharide was obtained the DMM group was no longer needed, so the authors removed it using a careful acid-base procedure, NaOH in Dioxane/Water (4:1) quenched with HCl until pH 3, followed by an acetylation reaction with acetic anhydride, affording the corresponding product in 48% yield. The next step consisted on the introduction of the lactyl moieties. Thus, the authors removed the acetyl groups using NaOMe with 74% yield, followed by treatment with used (*S*)-2-chloropropionic acid (after hydroxy deprotonation with NaH),

affording **II.20** in 46% yield. The final two involved the coupling with the pentapeptide in a 41% yield and the final hydrogenation to remove the benzyl groups, affording **II.21** in 76%.
The authors reported the synthesis of the tetrasaccharide **II.21**, in 18 steps and 0.04% yield. In this study the protective group for the amino moiety was the DMM moiety which is highly related to the previous phthalimide, and the leaving group at the anomeric position was once again the trichloroacetimidate group. This was the first report of the synthesis of a tetrasaccharide with the pentapeptide linked to the two O-3 lactyl groups.
In 2006 Fukase group reported the synthesis of several PGN fragments, mono-, di-, tetra- and octasaccharides with the penta-peptide assembled in order to understand how was the innate immune response triggered.[16] The synthesis relied on the same basis of a previous report from the same authors,[11] using Troc as the protecting group for the amine moiety and trichloroacetimidate group on the anomeric position. With these studies the authors determined that the minimal recognition pattern for hPGRP-L was the NAM unit linked to three amino acids. The authors also discovered that the NAM unit linked to two amino acids (MDP) was recognized by NOD2.

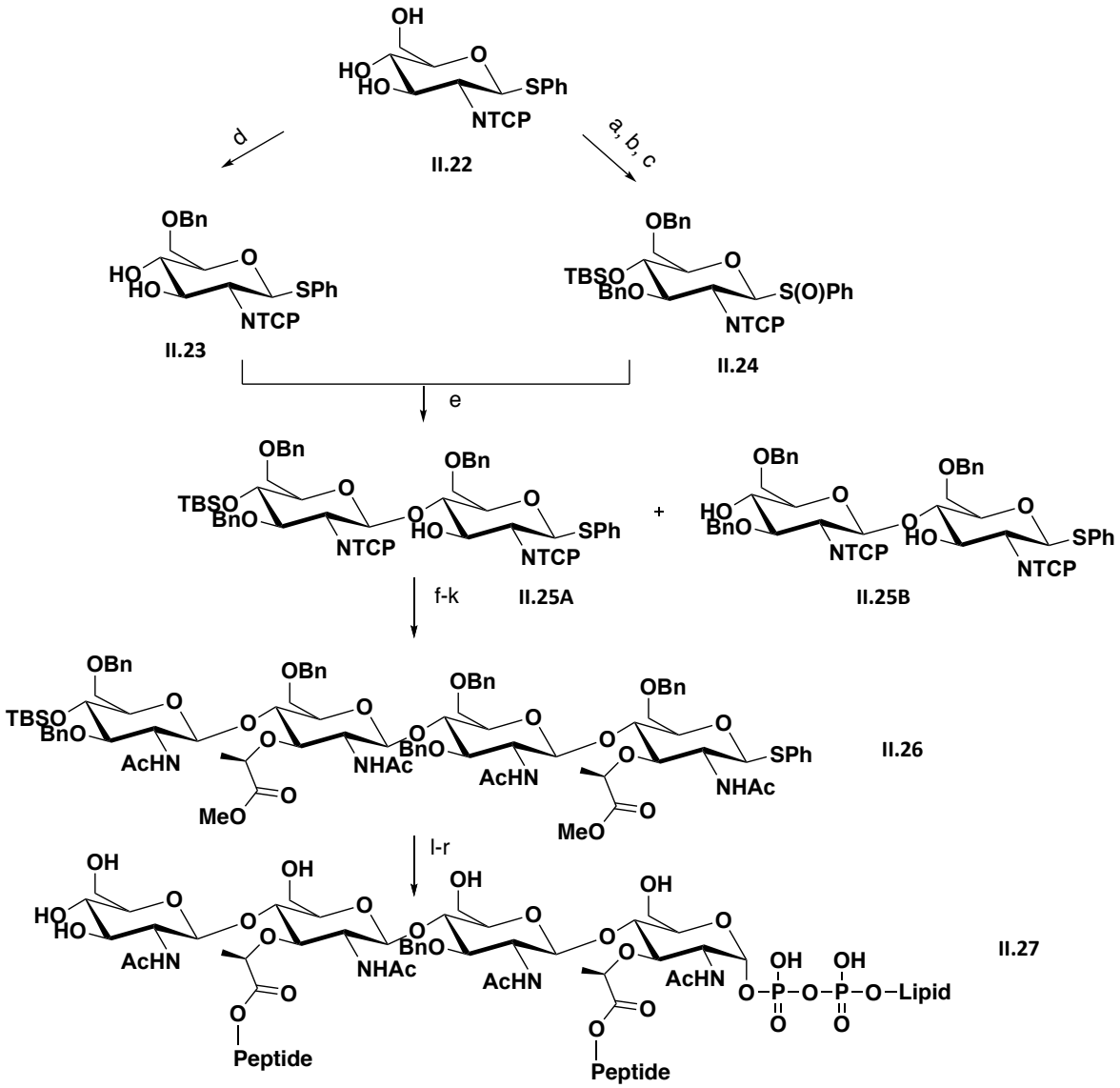


Figure 2.6 Synthesis summary to accomplish II.27 reported by Walker.[17] (a) Bu_2SnO, toluene, reflux, then Bu_4NI, BnBr, reflux, 51%; (b) TBSOTf, 2,6-lutidine, DCM, 91%; (c) *m*CPBA, DCM, -78 to -60 °C, 93%; (d) (i) $(Bu_3Sn)_2O$, MeOH, reflux; (ii) Bu_4NI, BnBr, toluene, 91 °C, 75%; (e) II.22 then (a) then *m*CPBA, DCM, -78 to -60 °C, 91%, then Tf_2O, DTBMP, ADMB, MS 4 Å, DCM, -60 to -30 °C, 75%; (f) *m*CPBA, DCM, -78 to -60 °C, 86%; (g) II.22 then (a) then mCPBA, DCM, -78 to -60 °C, 91%, then II.23 Tf_2O, DTBMP, ADMB, MS 4 Å, DCM, -60 to -40 °C, 58%; (h) products from f) and g) Tf_2O, DTBMP, ADMB, MS 4 Å, DCM, -40 °C, 77%. (i) $(NH_2CH_2)_2$, THF/ACN/EtOH (1:2:1), 60 °C; (j) Ac_2O, MeOH/H_2O (5:1), r.t., 75% two steps; (k) NaH, *S*-(–)-2- bromo-propionic acid, THF, 0 °C to r.t.; (l) $TMSCHN_2$, benzene/ MeOH (3:1), 0 °C, 70% two steps; (m) (i) NIS, ACN/H_2O (5:1), r.t., 75%; (ii) 1H-tetrazole, $(iPr)_2NP(OBn)_2$, DCM, -40 to -20 °C; (iii) mCPBA, DCM, -40 °C to r.t., 84% two steps; (iv) TBAF, THF, 0 °C to room temp; (v) lipid, 1.3 M KOH, THF/H_2O (10:1), r.t., 64% two steps; (n) HATU, DIEA, DMF, r.t., 60%; (o) $Pd(OH)_2/C$, H_2, 44%; (p) ammonium heptaprenyl phosphate, 1,1'-carbonyl diimidazole, THF, r.t., then tetrasaccharide, $SnCl_2$, DMF, r.t., 50%; (q) TBAF, DMF, r.t., 69%; (r) $(CH_3{}^{14}CO)_2O$, toluene/16 mM NaOH in MeOH (1:1), sonication, 37 °C, 50%.

In 2007 Walker and her co-workers reported the synthesis of the heptaprenyl-lipid IV (**II.27**), which consists on a tetrasaccharide $(NAG\text{-}NAM)_4$ linked to a diphospholipid at the anomeric position Figure 2.6.[17]

The authors started the synthesis with the common intermediate **II.22** that can be converted, in three steps, to the donor **II.24** as well as to the acceptor **II.23**, in one step.

To prepare **II.23** the first step consisted on the benzylation of the O-6 and O-3 hydroxy groups with Bu_2SnO and BnBr in 51% yield. Then the O-4 was protected with a silyl group by treatment with TBSOTf and lutidine, and isolated in 91% yield. The final step consisted on the oxidation of the thioglycoside, to become a good leaving group, with *m*CPBA affording **II.23** in 93% yield. To attain **II.24**, **II.22** was treated with $(Bu_3Sn)_2O$ in methanol followed by the addition of BnBr and TBAI allowed the monoprotection of O-6 hydroxy group with the benzyl group and **II.24** was obtained in a 75% yield. These two building blocks were produced in a gram scale amount. The next step was the glycosylation reaction. Tetrachlorophthalimide (TCP) group at the amino moiety, hindered the O-3 glycosylation reaction leading to a β-(1,4) glyosidic bond, despite of the O-3 free hydroxy group.

Reaction of **II.23** with **II.24** in the presence of Tf_2O, afforded **II.25A** in 75% yield. The construction of the tetrasaccharide was carried out by glycosylation with other disaccharide, **II.25B**. This second disaccharide **II.25B** was obtained using as donor a monosaccharide with benzyl group at the positions O-6 and O-3 and S(O)Ph at the anomeric position prepared from **II.22** and as acceptor **II.23**. The inverted addition of the donor to the acceptor **II.23** led to the formation of the disaccharide in 58% yield. The glycosylation of these two disaccharides (**II.25A** and **II.25B**) generated the desired tetrasaccharide in 77% yield. Since the tetrasaccharide was obtained the DMM group was no longer needed, so the authors removed it using hydrazine followed by N-acetylation with acetic anhydride in 75% yield for these two steps. This new tetrasaccharide has alternated O-3 positions free. The authors introduced the lactyl moiety using *S*-(–)-2-bromo-propionic acid after hydroxy deprotonation with NaH, followed by an O-alkylation step, affording **II.26** in 70% yield. The oxidation of the SPh group with NIS was done with 75% yield. Next the phosphate group was introduced at the anomeric position using $(i\text{-Pr})_2NP(OBn)_2$, followed by oxidation of the P-OBn groups with *m*CPBA in 84% yield for these two steps. The next two steps consisted on the TBS group removal, with TBAF, and hydrolysis of the lactyl ester with 1.3 M KOH in 64% yield for these two steps. The peptide coupling was next performed using HATU and DIEA in 60% yield. To accomplish **II.27** only four steps were left. First the benzyl groups were removed by hydrogenation in a 44% yield, then ammonium heptaprenyl phosphate was incubated with CDI and then added to the tetrasaccharide, to form the diphosphate linkage at the anomeric position, in 50% yield. The

remaining steps involved the deprotection of the peptide strand with TBAF in 69% yield and the coupling to the lipid strand to afford **II.27** in 50% yield.

In this report the authors established an orthogonal synthetic plan, based on the use of a building-block possessing the amino group protected with tetrachlorophthalimid (TCP) and thiophenol as the anomeric protecting group. In this study the authors used an enzyme, PBP1a, a peptidoglycan glycosyl transferase from *E. Coli*, in order to elongate the PGN strand, mimicking the natural process and in fact the authors successfully converted the synthetic tetrasaccharide into octasaccharide in 51% yield.

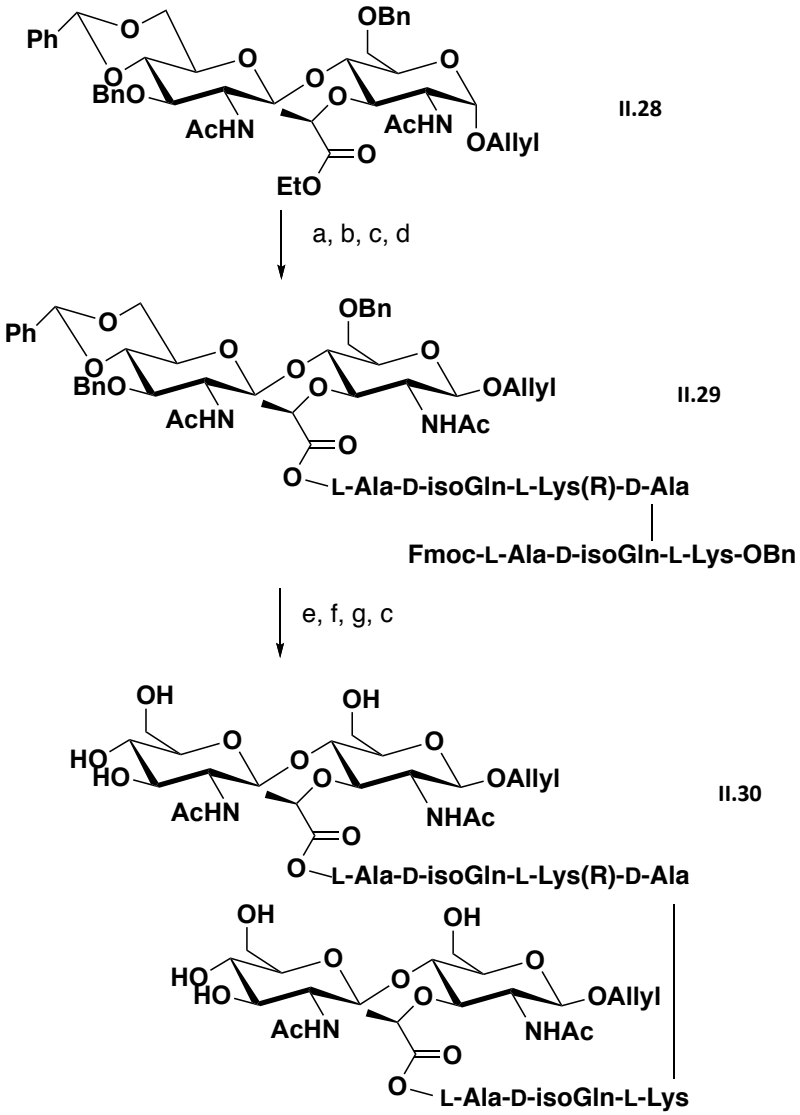


Figure 2.7 Synthesis summary to accomplish II.30 reported by Fukase.[18] (a) LiOH, THF/1,4-dioxane/H_2O = 4/2/1, quantitative; (Peptide: (b) TFA; (c) $HClEt_2O$;) (d) Peptide, HATU, Et_3N, DMF/DMSO = 1:1, 75% in three steps; (e) 10% TFA/DCM; (f) 20% piperidine/DMSO; (g) H_2,$Pd(OH)_2$, AcOH.

In 2009 Fukase and his co-workers reported the first synthesis of the cross-linked PGN fragments from *Streptococcus pneumoniae* cell wall.[18] The authors were able to use the

disaccharide **II.28** from previous reports[11, 19] as key intermediate in order to cross-link the peptide chains, Figure 2.7. Compound **II.28** was synthesized via glycosylation reaction with two monomers, **II.2** and **II.4**, Figure 2.3. Disaccharide **II.28** was hydrolyzed to the carboxylic acid and the peptide treated with TFA to remove the Boc protecting group. The coupling step was carried using the standard procedure with HATU, affording **II.28** in 75% yield. Next, the arylidene group was removed after treatment with 10 % TFA. The authors reported difficulties associated with removal of the Fmoc group, due to low solubility, but it was removed after treatment with 20 % piperidine. Next, the coupling with the second disaccharide was carried using the same conditions as before, affording the corresponding product in 42-26% yield depending on the lysine protecting group. The last step consisted on the hydrogenation reaction to remove all the benzyl groups, affording **II.30** in a quantitative yield. The authors investigated the biological activity of all the synthetic PGN fragments and found that the cross-linked PGN fragment is not the major ligand of human NOD2. Which in fact, can be due to the fact that PGRP might only recognize longer PGN fragments.

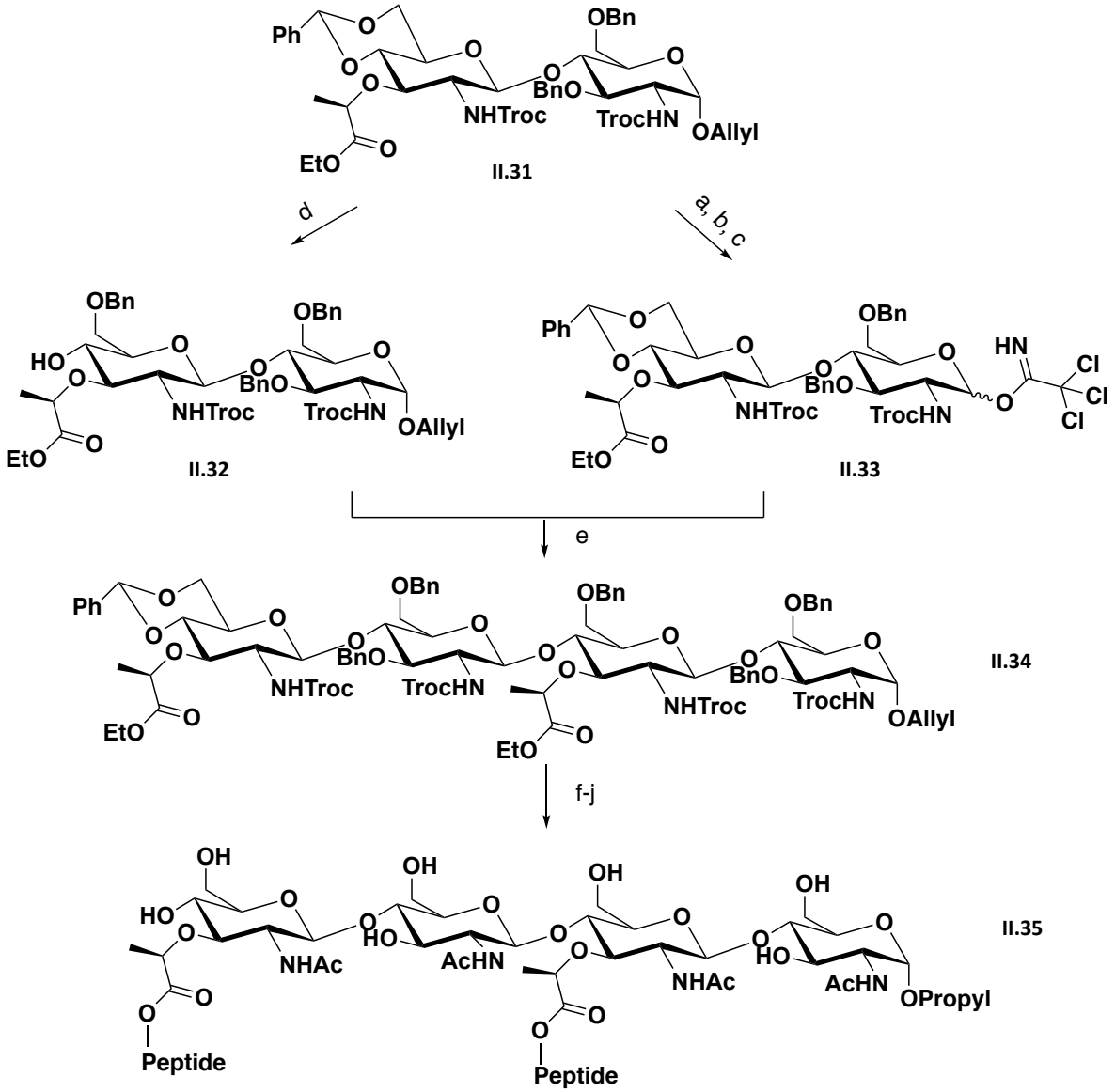


Figure 2.8 Synthesis summary to accomplish II.35 reported by Fukase.[20] a) [Ir(cod)H-($MePh_2P)_2$]PF_6, THF, 1.5 h; b) I_2/H_2O, 30 min, 81%; c) CCl_3CN/Cs_2CO_3, DCM, 30 min, quantitative; d) $Me_3N\,BH_3/BF_3{\cdot}Et_2O$, ACN, 1 h, 83%; e) TMSOTf, DCM, 158 °C, 4 Å MS, 40 min, 16%; f) Zn/Cu, AcOH,3 h, Ac_2O/Py, 2 h, 62%; g) LiOH, quantitative; Condensation: h) WSCD/HOBt/DMF/TEA, HCl·L-Ala-D-isoGln(OBn) or HCl·L-Ala-D-isoGln-L-Lys(Z)(OBn) or i) HATU/DMF/TEA, HCl·L-Ala-D-isoGln-L-Lys(Z)-D-Ala(OBn) or HCl·L-Ala-D-isoGln-L-Lys(Z)-D-Ala-D-Ala(OBn); j) $H_2/Pd(OH)_2$, AcOH.

In 2013 Fukase and his co-workers reported the synthesis of the tetrasaccharide $(NAM\text{-}NAG)_2$ (**II.35**), Figure 2.8, with different peptide chains, from di- to pentapeptide, in order to understand how the fragment sequence can affect the immune response.[20]

Compound **II.31** was synthesized via glycosylation reaction of two monomers; a donor with 4,6-O arylidene, O-3 lactyl ethyl ester, NHTroc and trichloroacetimidate at the anomeric position; an acceptor with O-6 and O-3 benzyl group, NHTroc and allylic alcohol at the anomeric position. After glycosylation reaction the compound **II.31** was obtained in 84% using TMSOTf as catalytic promotor. To produce the tetrasaccharide **II.34** the authors needed to

convert the **II.31** into an acceptor **II.32** and a donor **II.33**. To synthesize the acceptor **II.32** only one step was left, the regioselective opening of the arylidene ring to give the O-4 free position to perform the glycosylation. This reaction was carried using the standard conditions as used before by the authors, $Me_3N.BH_3$ in ACN for 1 hour, affording **II.32** in 83% yield. **II.33** was prepared in three steps from II.31: the isomerization of the allyl double bond, using an Ir complex; the hydrolysis assisted by iodine, in an 81% yield for these two steps; treatment with CCl_3CN to install the trichloroacetimidate group at the anomeric position, and **II.33** was obtained in a quantitative yield. Next, the glycosylation reaction was carried to afford **II.34**. The reaction using TMSOTf as promotor gave the tetrasaccharide **II.34** in a 16% yield. However, the authors found that if N-phenyltrifluoroacetoamidate was used as anomeric position group and increasing the ratio to 1:1.5 (donor/acceptor), the yield improved to 61%, with the same selectivity. Since the tetrasaccharide **II.34** was obtained, the Troc group was no longer needed, and was removed using a Zn/Cu in AcOH, for 3 hours followed by an acetylation step, (acetic anhydride in pyridine for 2 hours), affording the N-acetylated product in 62% yield. The saponification of the ethyl esters with LiOH afforded the corresponding product in a quantitative yield. The following steps involved the condensation of the tetrasacchardie with a library of peptides, di-, tri-, tetra- and pentapeptide. Thus, different protocols were used depending on the peptide strand. The final step to obtain **II.35** consisted on the hydrogenation to remove all the benzyl groups, which depending on the peptide afforded the **II.35** in 53-825 yield.

In fact, this study showed that human NOD2 activation by PGN fragments depends on the circumstances under which PGN is hydrolysed by enzymes, N-glucosamidases or muramidases. Since both di- and tetrasaccharide can activate de human NOD2 factor. Regarding to the synthesis in PGN fragments this report is based on previous ones from the same authors[11, 16, 18, 19, 21-23] but constitutes the first synthesis of NAM-NAG oligosaccharide from Fukase's group.

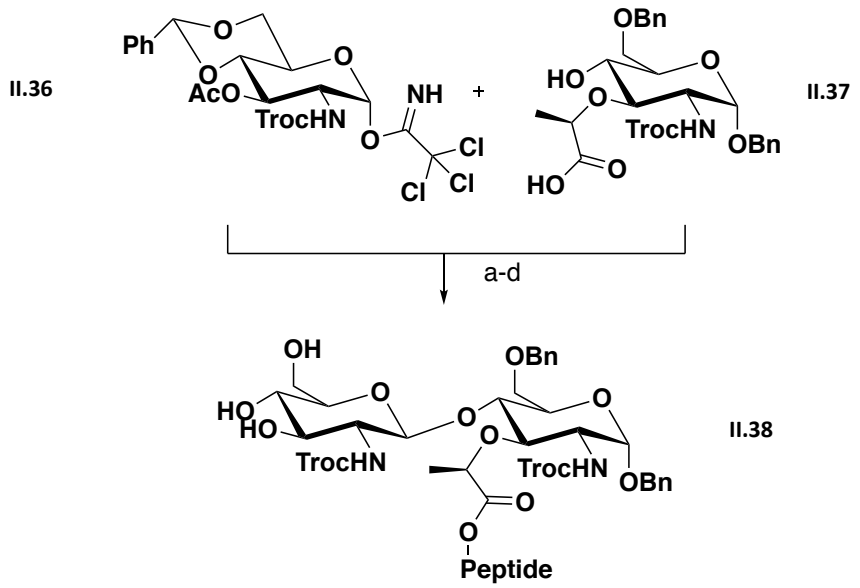


Figure 2.9 Synthesis summary to accomplish II.38 reported by Marques.[24] a) TMSOTf, DCM, 3 Å MS, -15 °C, 52%; b) Zn-Cu, THF: AcOH: Ac_2O (1:1:1), then Ac_2O/pyridine, 63%; c) LiOH, THF:1,4-dioxane:H_2O, 69%; d) (L-Ala-D-isoGln-L-Lys(Ddiv)-D-Ala-D-Ala) [Ddiv (1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl)], HMPB-AM, TFA 1% DCM.

Our group, in 2014, reported a synthesis of a fully deprotected disaccharide NAG-NAM in 12 steps, Figure 2.9.[24] The synthesis started by preparation of the donor **II.36** by using N-Troc glucosamine.. The first step consisted on the formation of the O-4,6 arylidene and O-3 and O-1 acetylation. Thus, N-Troc glucosamine was treated with benzaldehyde dimethyl acetal and $ZnCl_2$ overnight, the crude dissolved in pyridine and acetic anhydride and the product was afforded in 78% yield after these two steps.

The authors selectively removed the acetyl group from the anomeric position using morpholine in ethyl acetate in 87% yield. The final step consisted on the preparation of the trichloroacetimide at the anomeric position using CCl_3CN/Cs_2CO_3, in dichloromethane for 2 hours affording **II.36** in 74% yield. To prepare the acceptor **II.37** the authors started with the N-Alloc glucosamine. The first step consisted on the benzylation of the anomeric position using BnOH and AcCl in 61% yield. Then the 4,6-O arylidene formation was attempted using benzylidene with $ZnCl_2$ in a 72% yield. Next, the lactyl moiety was introduced by using trifluoromethanesulfonyl-L-(*S*)-2-propionic acid ethyl ester and the product was isolated in 68% yield. Then $Pd(PPh_3)_4$ was added to remove Alloc group followed by in situ N-protection with the TrocCl to afford the corresponding product in 82% yield. The last step consisted in the selective arylidene ring opening using the classical procedure with $Me_3N.BH_3$, affording **II.37** in 72% yield. The glycosylation step was carried using TMSOTf as promoter giving the product, with the desired selectivity, a β-(1,4) glyosidic bond in 52%. Since the disaccharide

was obtained the Troc group was no longer needed, and it was removed using a Zn/Cu in AcOH: THF: AC_2O followed by an *in situ* N-acetylation reaction, using acetic anhydride in pyridine for NHAc, product in 62% yield. Next, the lactyl ester and O-3 acetyl group were hydrolyzed by treatment with LiOH, in 69% yield. Having this product in hands the authors followed two routes: a) a fully deprotection route to afford NAG-NAM unit; b) coupling with a peptide. In order to fully deprotect the disaccharide a hydrogenation reaction was performed and the NAG-NAM product was isolated in a quantitative yield. The peptide was previously synthesized via Fmoc solid-phase synthesis, using HMPB-AM resin. After coupling the peptide and cleaving the product from the resin **II.38** was obtained.

In this study the protecting group at the amine moiety was the Troc group and trichloroacetimidate group was used on the donor unit. The benzyl group at the anomer position of the acceptor unit was a key group in order to facilitate the protecting groups removal in one step. The previous reports[1, 2, 25] gave us the know-how need to provide a simple and driven synthetic route towards NAG-NAM moieties.

In 2016 Fukase and his co-workers reported a chemoenzymatic approach to the large scale synthesis of a *meso*-DAP type PGN from *Mycobacterium*.[26] Since the *meso*-DAP has been reported to be the most potent activator of Nod2 there was a need to develop a large scale synthesis protocol. The use of L-aminocyclase and D-aminocyclase was a key factor to be able to afford the *meso*-DAP in large scale in order to proceed with the biological studies.

PGN synthesis is a challenging task. The problematic aspects of all these synthetic plans are: i) the choice of the nitrogen protecting group; ii) the choice of the anomeric protecting group; iii) one-pot procedures are favorable, allowing to avoid some synthetic steps. The acetyl group as N-protecting group presents several limitations to used due to its poor solubility, low reactivity and problems when it came to glycosylation reactions.[1]

2.1.3 Synthesis of chitooligosaccharides and other glucosamine congeners

Chitooligosaccharides (COSs) have attracted attention of the scientific community. The use of COSs has also attracted the attention of the scientific community due to their innumerous biological activities.[27, 28] Fascinatingly, COSs can present different degree of polymerization (DP), degree of N-acetylation (DA) and pattern of N-acetylation (PA) and consequently trigger different biological responses.[29] COSs derivatives have also played a critical role in a variety

of biological processes, for example Nod factors, also called lipochitooligosaccharides, are nodulation signal molecules in the symbiosis of Rhizobium bacteria and leguminous plants.[30] Another example is the TMG-chitotriomycin that possesses an N,N,N-trimethyl-D–glucosamine (TMG) residue linked to the non-reducing end of a chitotriose, which is a potent and selective inhibitor against the β-N-acetylglucosaminidases (GlcNAcases) of insects and fungi, however does not inhibit GlcNAcases of plants and mammals.[31, 32] An account by Yu and Yang covers the recent advances on the synthesis of COSs and congeners.[3] COSs can be achieved by chemical or enzymatic degradation of chitin or chitosan. However, this route leads to heterogeneous mixtures and currently, hydrolysis of chitin is used to form mainly glucosamine monomer. Access to COSs with size-defined molecules with distinct substitution patterns is significantly desirable. However, the N-acetyl group presents a major limitation to preparation of COSs and derivatives due to, not only the poor solubility, but also the role of N-protecting group on the enantioselective glycosylation as reported by our group and others.[1, 24] Recently, Beau *et al.* have reported an elegant strategy to prepare bioactive COSs and conjugates, in particular lipochitooligosaccharides, were chitin acetolysis was applied to achieve a controlled depolymerization.[33] Indeed the formation of well-defined COS allows unequivocally elucidation of the structure and activity relationships of COS. Total synthesis of COSs with defined oligomeric sizes and substitution patterns can be achieved, however it is challenging and is still a demanding task.

COSs have an important role in several proteins-COSs interactions.[34] Carbohydrate recognition is essential for cell adhesion, growth and signaling in all living organisms. LysM, is a highly conserved carbohydrate binding module, found in proteins from viruses, bacteria, fungi, plants and mammals. LysM domains are carbohydrate-binding protein modules, which recognize polysaccharides containing GlcNAc residues, however the mechanism of their binding to COSs has been poorly explored. The regioselective strategies already developed for chitin and chitosan modification might also be useful to enable the synthesis of COSs derivatives, paving the way to new discoveries in the field of carbohydrate-protein interactions.

In this field Beau group has been the pioneer developing many methodologies in order to synthesize modified COSs.

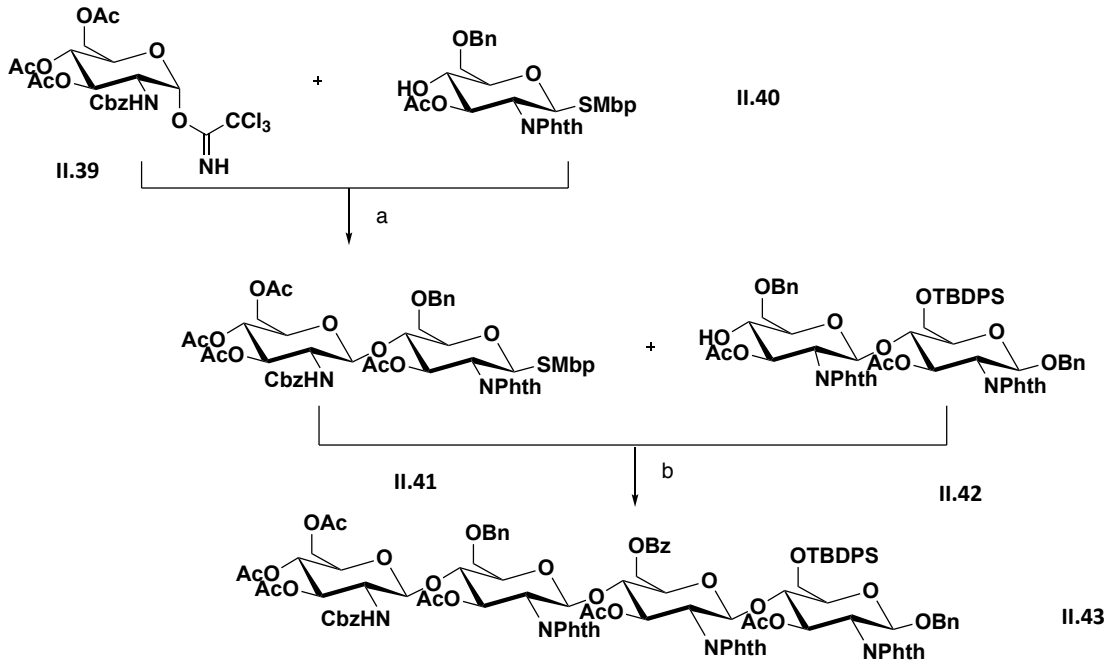


Figure 2.10 Synthesis summary to accomplish II.43 reported by Beau.[35] a) II.40 2 equiv., II.39 1 equiv., BF_3Et_2O (0.5 equiv.), PhMe, 7h, -78 °C – r.t., 76%. b) toluene, MS 4 Å (1 h, r.t.), then NIS (1.2 equiv.), TfOH (0.2 equiv.), 3 h, –30 °C, 61%.

In 2013 Beau and his co-workers reported a methodology to synthesize a tetrasaccharide **II.43** starting with a proper functionalized glucosamine derivatives, **II.39**, Figure 2.10.[35]

The authors started the synthesis with the glycosylation reaction of **II.40** with **II.39**, in toluene promoted by $BF_3.Et_2O$. The authors found that when this reaction was carried in DCM the yield was low (23-38%). The same was observed when the reaction was carried in toluene at 0 °C (68%). This was maybe due to an oxazolidinone formation with participation of the carboxybenzyl (CBz) group. Once optimized, this reaction led to the **II.41** in 76% yield. To produce **II.42** the authors performed a glycosylation reaction between a donor, 4,6-O arylidene, O-3 acetyl, NPhth and 2-methyl-5-*tert*-butylphenylthiol (SMbp) at the anomeric position; an acceptor, O-6 TBDPS, O-4 free, O-3 acetyl, NPhth and O-Bn at the anomeric position. This reaction was carried out using NIS (2.5 equiv.) as oxidant, TfOH (catalytic) in DCM at -10 °C in 97% yield. The authors screened the reaction conditions to obtain the best glycosylation reaction and enantioselectivity. They realized that the same solvent modification from DCM to toluene resulted in a yield improvement from 26 to 61% (**II.43**), using 1:1.1 ratio (**II.41** : **II.42**).

The use of different protecting groups allows the selective deprotection which means selective modification of several moieties. In this study the TBDPS group was removed in

order to sulfatate the hydroxy group, that was latter linked to a fatty acid chain. Both carboxybenzyl (Z) and phthalimide groups were removed followed by acetylation of the amino group. The strategy used is similar to the one used in the synthesis of PGN fragments and orthogonal protecting group strategy and regioselective modifications.

In 2014 Beau group reported a breakthrough methodology, consisting on glycosylation reactions using peracetylated glucosamine (**II.44**) as donor catalysed by iron triflate salts, Figure 2.11.[36]

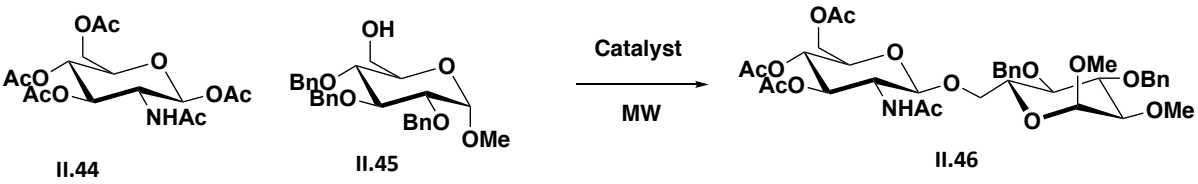


Figure 2.11 Synthesis summary to accomplish II.46 reported by Beau.[36]

The authors compared the effect of two different catalysts, $Fe(OTf)_3$.6.2DMSO and $Fe(OTf)_3$. in the glycosylation reaction, mediated by microwave radiation. They found that the use of $Fe(OTf)_3$ led to a higher yield (93%) when 2 equiv. of 2,4,6-tri-*tert*-butylpyridine were added at 110 ^oC for 45 min. The same conditions using $Fe(OTf)_3$.6.2DMSO led to a 89% yield. They also found that the temperature was a key to improve the yields since when it was not applied the yields were very low (12-38%) and the reaction would take up to 96 hours. The authors also tried the same reaction without microwave which in fact did not occurred. Other Fe salts were investigated, but did not have the same efficiency. It was found that if the reaction temperature was slightly lower (80 ^oC) $Fe(OTf)_3$.6.2DMSO, the yield observed, 92%, was the same as with $Fe(OTf)_3$.

Using $Fe(OTf)_3$.6.2DMSO, as catalyst, in 15% mmol the authors were able to perform the glycosylation reaction using **II.44** as donor in 92% yield with one single anomer. The authors went further and screened a library of acceptors in order to understand the limitations of this method.

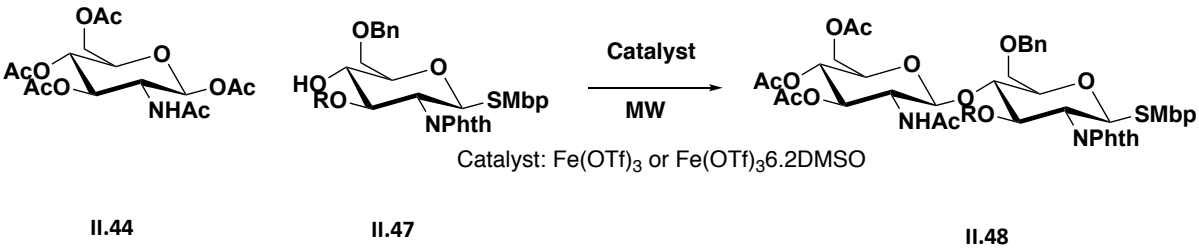


Figure 2.12 Synthesis summary to accomplish II.48 reported by Beau.[36] R= acetyl or benzyl.

The same procedure was explored using a protected glucosamine, **II.47**, as acceptor, and it resulted in a 25-27% yield of glycosylation reaction, Figure 2.12.

In 2014 the same group reported a one-pot methodology that consists on the regioselective protection, benzylation and 4,6-O arylidene formation, followed by glycosylation of two fully protected glucosamine derivatives, Figure 2.13.[37]

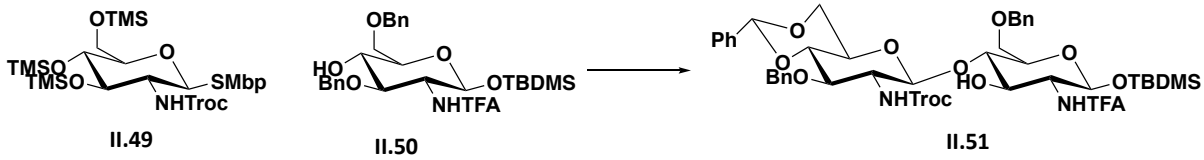


Figure 2.13 Synthesis summary to accomplish II.51 reported by Beau.[37] Conditions: PhCHO (2.2 equiv.), Et_3SiH (1.1 equiv.), TfOH (10%), 3 Å MS, DCM, then II.50 (0.8 equiv.), NIS (1.5 equiv.), 73%.

Beau group established a procedure catalyzed by triflic acid to protect, in a one-pot manner, all the sylilated hydroxy groups, and then realized that the benzylation conditions were compatible with the conditions of the glycosylation reaction. With this one-pot procedure the disaccharide **II.51** was afforded in a 73% yield starting with the full TMS glucosamine **II.49** (Figure 2.13). This report is remarkable since it allows many modifications in one single step. Also, the use of catalytic triflic acid and MS can be very interesting when it comes to large scale processes or even flow chemistry.

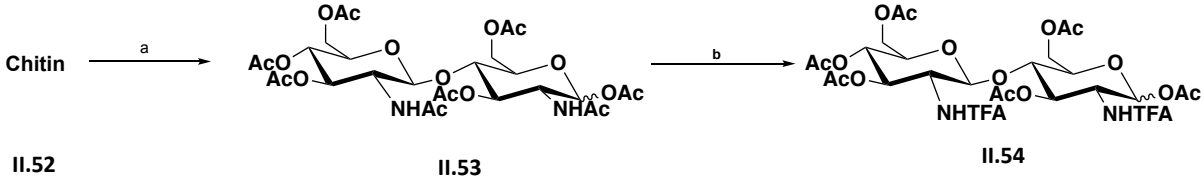


Figure 2.14 Schematic representation of NHTFA oligosaccharides synthesis starting with chitin. a) Ac_2O, H_2SO_4, 50 °C, 24 h; b) TFAA, pyr, 120 °C, 20 min.

The same year Beau group also published a synthetic route of N-acetylglucosamidases inhibitors, using a well-defined COS.[33] In this publication Beau and his co-workers reported a procedure that allows the acetolysis of chitin (**II.52**) leading to size defined full acetylated oligosaccharides (DP = 1, 2, 3, 4). Then a *trans*-N-acetylation step occurs providing N-TFA well defined oligosaccharides, Figure 2.14.

As stated previously, one of the most challenging steps is the presence of the N-acetyl moiety due to the low solubility and reactivity. Thus, this Beau's methodology would allow a simpler manipulation of the substrate, since the N-trifluoroacetamide (NTFA) group enhance both

solubility and reactivity. In this paper the construction of COSs undergoes on chitin hydrolysis instead of glycosylation of glucosamine building blocks.

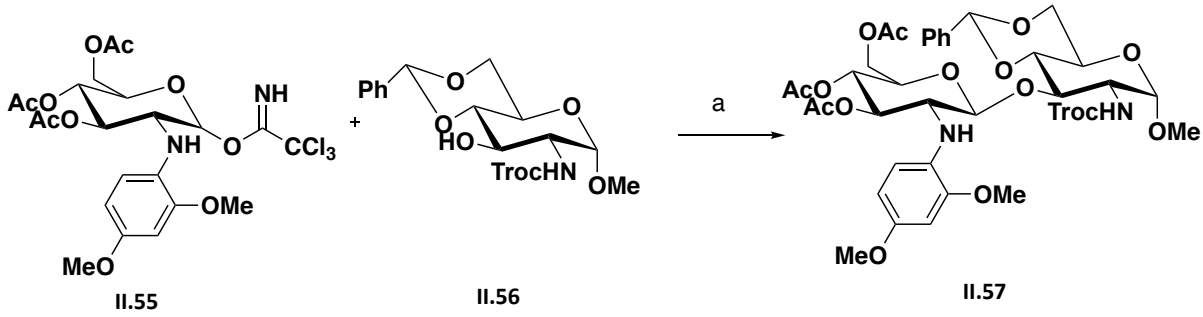


Figure 2.15 Synthesis summary to accomplish II.57 reported by Fukase.[38] a) TMSOTf, 4 Å MS, DCM, -20 ºC, 2 h, 90%.

In 2017 Fukase group reported a methodology for a glycosylation reaction using **II.55**, as starting material (Figure 2.15).[38] The authors started by screening the optimal conditions using different donors and BnOH as acceptor. They found the donor **II.55** has the best β selectivity (49:1), 95% yield when glycosylated with BnOH.

In matter of fact this new procedure is oriented by the nitrogen protecting group, an aryl group that can be installed through a Buchwald-Hartwig reaction. Glycosylation reaction catalysed by TMSOTf allows the O-3 glycosylation, Figure 2.15, in 90% yield only affording the β anomer. This report proved that with the right donor, even a hindered acceptor can provide high yields and selectivity.

Synthesis of NAG containing oligosaccharides,[3, 39-41] such as NAG-NAM disaccharide, is a challenging task due to the well-known limitations associated with the enantioselective glycosylation of NAG moieties: i) the presence of a β-(1,4) glycosidic bond requires a multi-step synthetic sequence to obtain a regio- and stereoselective assembly of glycosidic bonds, crucial for biological activity; ii) the use of 2-acetamido-2-deoxyglycosyl donors, due to the formation of 1,2-O,N-oxazoline intermediates,[1, 42-44] and the use of corresponding acceptors that are poor nucleophiles.[45]

Since one of the most difficulties on the PGN synthesis is the number of synthetic steps and the protecting group manipulation, as identified previously, a synthetic plan with fewer steps and involving one-pot methodologies would constitute a major advance on carbohydrate synthesis.

The COS production topic has been a review topic for the last years.[3, 46]

2.2 Results and Discussion

2.2.1 Goals

As demonstrated on the previous sections the traditional approaches for the synthesis of PGN fragments relays on the use of a fully protected monosaccharide, which is further glycosylated in order to elongate the carbohydrate chain.

These traditional approaches, Figure 2.16, have several difficulties: i) a proper N-protecting group, compatible with the synthetic steps, that enables enantioselective control over the glycosylation as well as the regioselective insertion of the lactyl moiety at O-3 of a modified NAG unit; ii) a proper anomeric group for the donor unit; iii) protecting groups at the acceptor unit that allow the regioselective β–(1,4) glycosydic bond in high yield. Usually these synthetic strategies involve many steps to produce the disaccharide NAG-NAM, and even more to obtain more complex structures.

Figure 2.16 shows the synthetic strategies towards the preparation of versatile disaccharides precursors of both NAG-NAM and NAM-NAG disaccharides already reported.

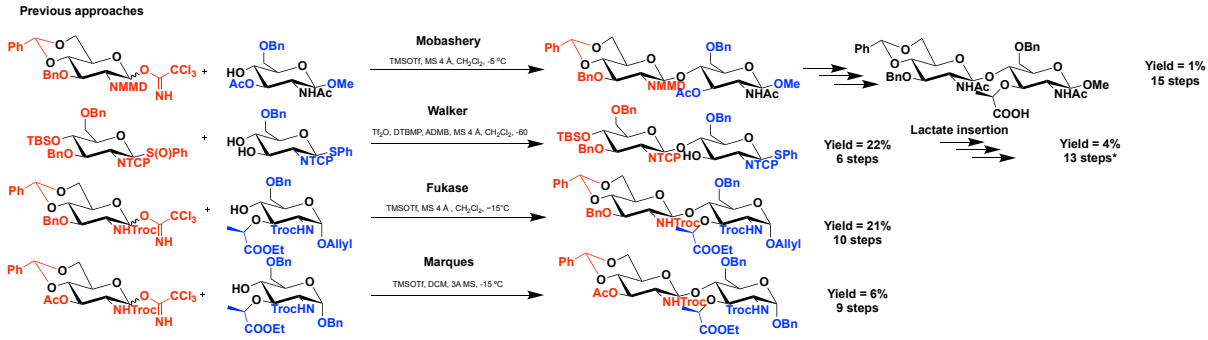


Figure 2.16 Schematic summary of the previous reported synthesis towards NAG-NAM containing oligosaccharides.

Since this work was developed in the context of a sustainable chemistry PhD program, the aim was to investigate a synthetic route considering the green chemistry principals. Thus, the main objective was the use of renewable resources as starting material; one-pot procedures and reduce the number of synthetic steps. Since our group had know-how on the synthesis of PGN fragments, the main goal of this work was to prepare PGN fragments using as starting material a natural polymer which already possesses the β–(1,4) glycosydic bond. Taking advantage of the already stablished β–(1,4) glycosidic bond on chitin (**II.52**), it would allow to

synthesize PGN fragments without the usual selectivity concern upon the glycosylation reaction.

Simultaneous to our work Beau group reported an approach to access well-defined size oligosaccharides from chitin (**II.52**) via the formation of peracetylated chitooligomers.[33]

Beau group obtained peracetylated chitooligomers by treating chitin with acetic anhydride and sulphuric acid.[33]

Then, using this chitooligosaccharide derivative and via a regioselective orthogonal synthesis, a fully protected chitobiose derivative could be achieved, Figure 2.17. One of the major difficulties of this synthetic plan, consists on the chemical differentiation of the three secondary hydroxy groups. Therefore, when PG4 and PG5 are the same protecting group a NAG-NAM precursor can be obtained, Figure 2.16.

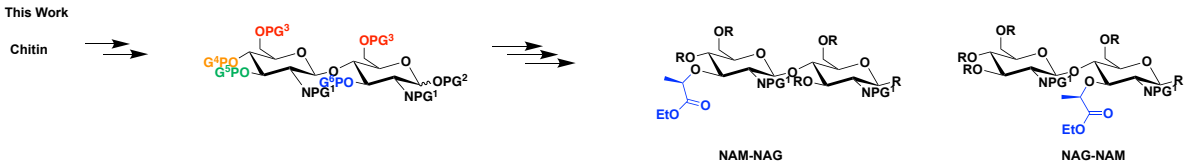


Figure 2.17 Schematic synthetic plan towards NAG-NAM containing oligosaccharides.

2.2.2 Preliminary studies: O-3 regioselective modification on chitobiose derivative

The aim of this work was to prepare PGN fragments, NAG-NAM and/or NAM-NAG containing oligosaccharides, some preliminary studies were undertaken in order to establish the proper synthetic plan.

In the last five years Minnaard group reported a very interesting approach in order to selectively modify one particular hydroxy group from several sugar units.[47-50]

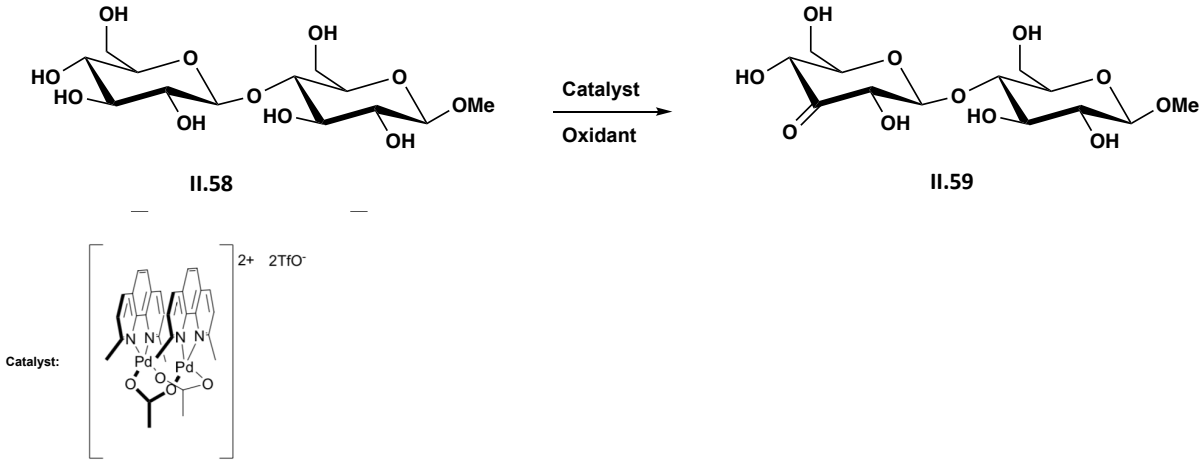


Figure 2.18 Regioselective hydroxy group oxidation catalyzed by palladium.

This methodology allows the differentiation of the hydroxy group (O-3) of a disaccharide where only one of the O-3 is oxidized, Figure 2.18.

This reaction was reported using a wide range of glycosides, N-acetyl glucosamine, glucose disaccharides[47] and in a maltoheptaosyl.[48,51]

This strategy takes advantage of a previous work from Waymouth on the oxidation of glycerol to dihydroxy acetone.[52] Thus, the selective oxidation of the O-3 position in a NAG-NAG disaccharide would allow the desired discrimination between the two units and install the lactyl moiety at the O-3 of the terminal unit creating a NAM-NAG precursor.

Inspired by Minnaard's group latest reports[47-51, 53] we envisaged to apply a similar procedure and, after the ketone formation, all the remaining hydroxy groups could be protected with the same protecting group, Figure 2.19. This synthetic route was designed in collaboration with Prof. Joachim Thiem during a three months stage in his lab.

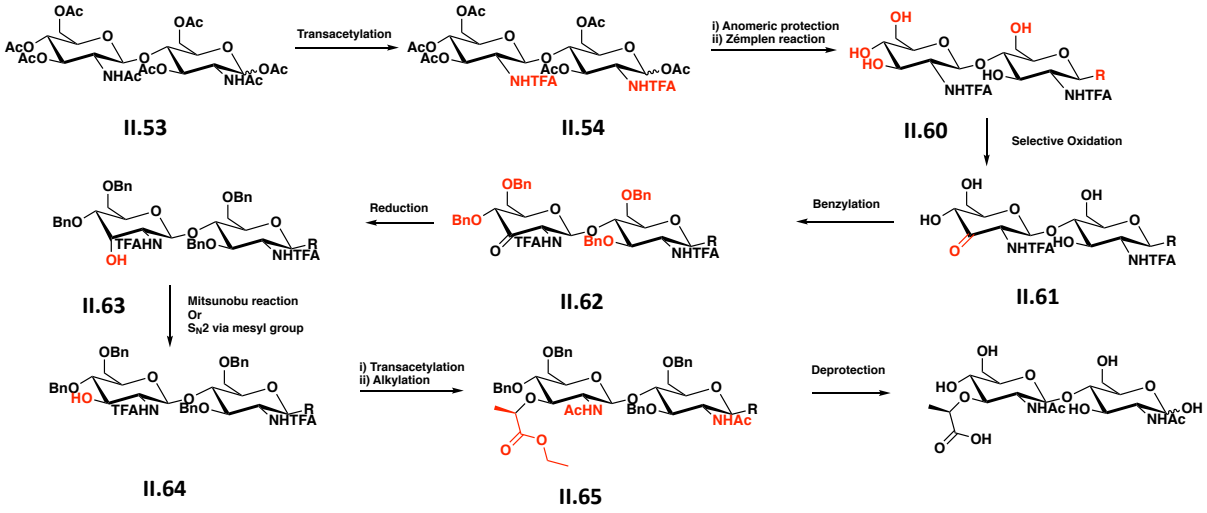


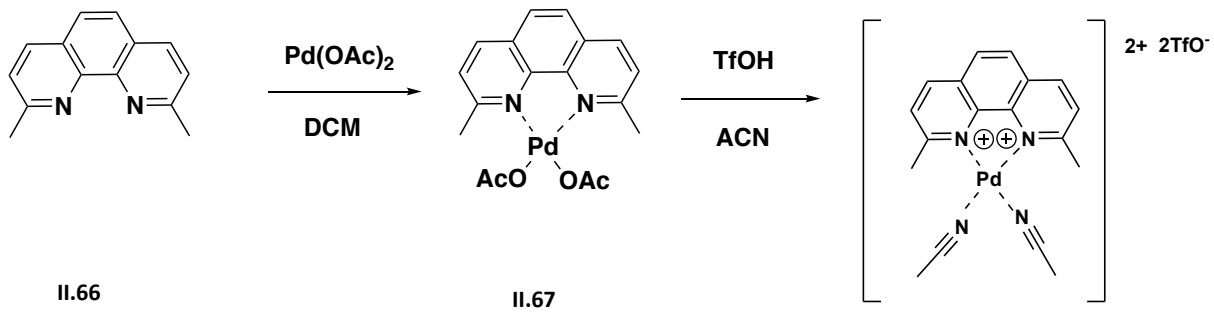
Figure 2.19 Proposed synthetic route to achieve a NAM-NAG precursor.

So a synthetic route was proposed starting with the peracetylated chitobiose (**II.53**) and perform the *trans*-N-acetylation to obtain the compound **II.54**, by the same procedure already described.[33]

We have used the procedure described by Beau *et al.*[33] to obtain the peracetylated chitooligomers, that can be purified by a common work-up procedure and separated by flash column chromatography, giving the desired peracetylated chitobiose **II.53** in 10 g scale amount, Figure 2.14. This reaction was carried by diluting portion wise 60 g of chitin (**II.52**) in an ice-cold mixture of 300 mL of acetic anhydride and 30 mL of sulphuric acid. The reaction was heated to 55 ^{o}C and let to stirred for 3 hours. Then the reaction was let to stirred for 16 hours at room temperature, after was heated to 55 ^{o}C for one more hour. The reaction was poured into an ice-cold solution of sodium acetate (120 g) in water (1.2 L). The resulting solid was filtered off. The filtrate was extracted with dichloromethane (3 x 1.5 L). The residue was purified by silica gel chromatography, 1 Kg of silica (DCM: acetone – 10:1 to 1:10), affording **II.53** in 10 g, the trisaccharide in 3 g and the tetrasaccharide in 1.5 g. The identification was made by TLC (1:1 DCM:acetone), stained with H_2SO_4 10% in methanolic solution, and ^{1}H NMR were anomeric protons and the acetyl groups integrals were the key to identify the DP.

It was proposed to use an anomeric protecting group capable of resisting through the synthesis and that could be used in glycosylation reactions, followed by methanolysis to produce **II.60**. Compound **II.60** would fulfilled the requirements for the O-3 oxidation, since it has a protecting group at the anomeric position, and also all the hydroxy groups exposed.

The oxidation of the **II.60** by Minnaard's methodology[47, 48] would provide the ketone **II.61**. After this step the remaining hydroxy groups need to be protected, so it was proposed to use the benzyl group (**II.62**), as protecting group. This would allow the simultaneous removal of protecting groups via palladium catalyzed hydrogenation in a more advanced stage. Compound **II.62**, requires inversion of the stereochemistry at C-3 position. Thus, it was anticipated that a common ketone reduction with $NaBH_4$ (**II.63**). This reaction would give the hydroxy group with the undesired stereochemistry.[47] To invert the stereochemistry, was proposed to perform a Mitsunobu reaction or a S_N2 reaction since the hydroxy group could be protected with the MsCl to obtain **II.64**. The conversion of **II.64** to the final product was already described, via lactyl insertion followed by removal of the phthaloyl group and N-acetylation. After all these steps, the compound **II.65**, would act as a NAM-NAG precursor.



II.66 **II.67** **II.68**

Figure 2.20 Synthesis of the catalyst for the oxidation reaction.

Initially the oxidation step was investigated on substrate **II.69**. The catalyst was prepared previously, according to Minnard's procedure, Figure 2.20.

The catalyst was prepared according to the literature procedure,[53-55] that consisted on the reaction of palladium diacetate with neocuproine (**II.66**) in DCM, overnight to afford **II.67**, Figure 2.19. The product formation was confirmed by ^{1}H-NMR, in 17% yield. Next **II.67** was treated with TfOH in an ACN solution, for one hour. This reaction afforded **II.68** in 88% yield, confirmed by ^{1}H NMR. The catalyst is a dimer constituted by **II.67** and **II.68** in equal amounts. The oxidant species can be oxygen,[53] benzoquinone[47] or 2,6-dichloro-1,4-benzoquinone.[47]

The synthesis started using the monosaccharide **II.69** as model, Figure 2.21.

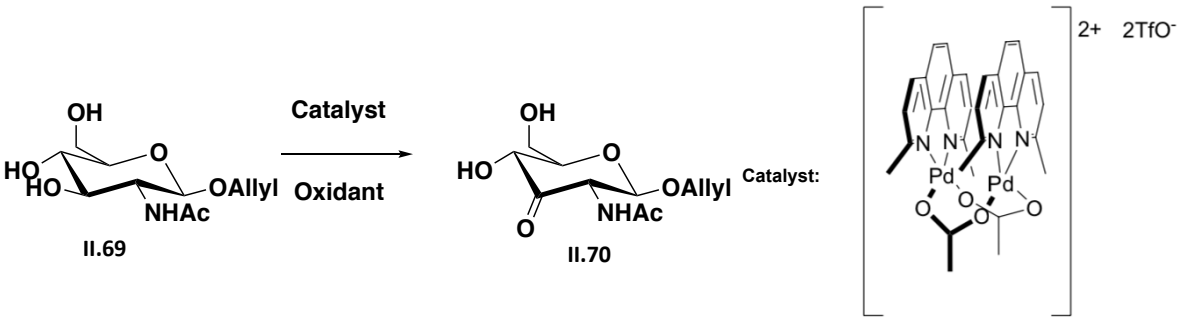
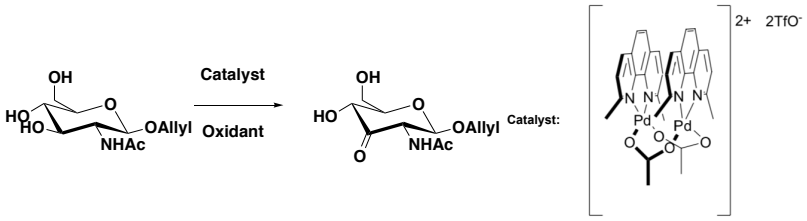


Figure 2.21 Monosaccharide O-3 oxidation reaction.

The O-Allyl group was chosen as anomeric protecting group, since this reaction is always reported on substrates with resistant groups at the anomeric position.

Table 2.2 Optimization of the reaction conditions for the oxidation at O-3 of II.69.



Entry	Catalyst (mol %)	Solvent	Oxidant	Temp (°C)
1	2.5	ACN:H_2O (10:1)	Benzoquinone (3 equiv.)	r.t.
2	2.5	ACN:H_2O (10:1)	Oxygen	r.t.
3	2.5	ACN:H_2O (10:1)	Oxygen[a]	r.t.
4	6[b]	ACN	Oxygen Diisopropyl phenol	60

20 min reaction; [a] A double balloon was used; [b] gradual additions of 2 equiv. every two hours

The experiments were based in the original procedure.[47] Before each reaction the catalyst was generated *in situ* by adding the **II.67** and **II.68** in the solvent. When the solids were dissolved in the solvent, the oxidant and the substrate were added, and the reaction was stirred overnight. Table 2.2 shows all the conditions tested, for the **II.69** substrate. In entry 1 the reaction was performed using the conditions reported.[47] However, after four hours of reaction the TLC showed no sign of the **II.70** product. Due to the results obtained the oxidant specie was changed to molecular oxygen, entry 2. After an overnight reaction the TLC showed

no sign of the **II.69** product again and the ^{13}C-NMR confirmed the TLC observation. It was decided to perform the same conditions using a double oxygen balloon in order to guarantee that the oxygen was present, entry 3. Once again, the **II.70** was not observed. Next an hybrid method was tested, consisting of, diisopropyl phenol[54] as co-oxidant instead of water, the reaction temperature was raised to 60 °C and the catalyst load increased to 6 mol%, adding 2 mol% every two hours. This last entry did not show any trace of **II.70**. Despite of the results observed with the trials on the monosaccharide **II.69** the disaccharide **II.71** was investigated since the anomeric group and also the nitrogen protecting group were different (**II.71**), Figure 2.22.

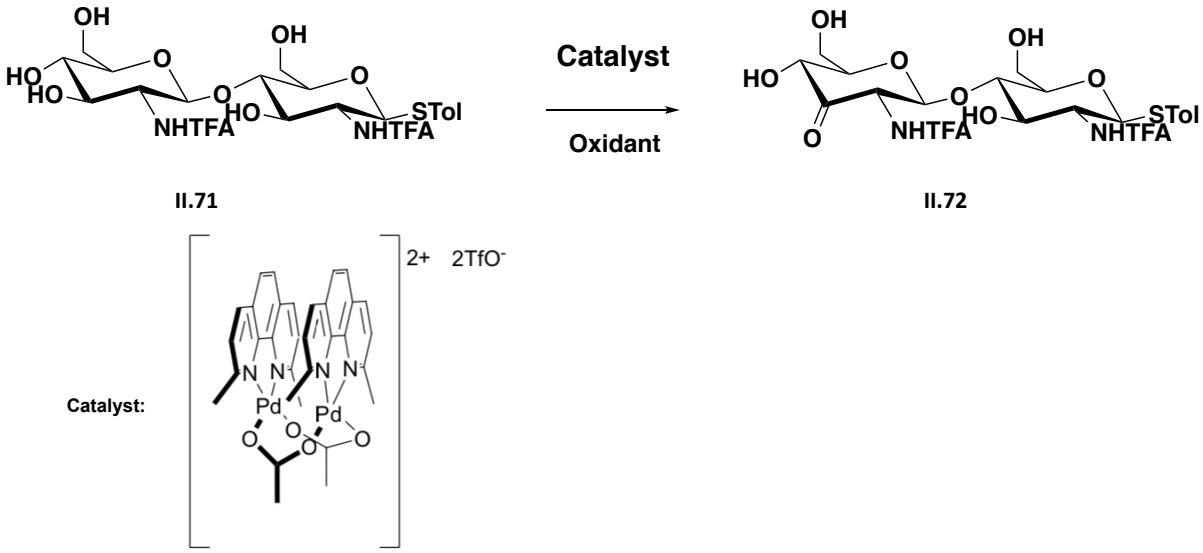


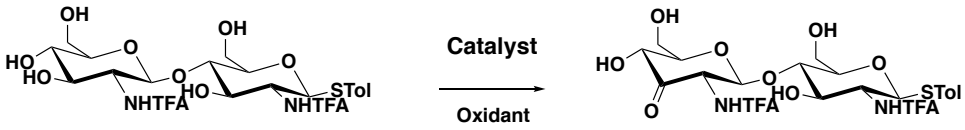
Figure 2.22 Disaccharide O-3 oxidation reaction.

Since Minnaard group reported the use of this catalyst (**II.67** : **II.68**) to oxidize sugars with sulphur groups at the anomeric position with no sign of oxidation of the sulfur, we decided to investigate this approach.[47]

The procedure was the same used before on the monosaccharide trails. Table 2.3 summarizes all the experiments, for the substrate **II.72**. In entry 1 the reaction was carried according to the reported procedure[47]. However, after four hours of reaction the TLC showed no trace of the **II.72** product. Since the disaccharide had a lower solubility when compared to the monosaccharide, the isolation procedure was revised. Thus, the crude of the oxidation reaction was treated with TBDMSCl, entry 2. Despite all the efforts no product was observed. The integrity of the catalyst was questioned during the reaction and so, on entry 3, the

reaction was protected with aluminum foil, and once again no product was observed. The last trial with the disaccharide was performed using a higher loading of catalyst, 6 mol% adding 2 mol%every two hours, and changing the solvent to DMSO:Dioxane, in order to promote a higher solubility of the product. Nevertheless, no product was observed.

Table 2.3 Disaccharide optimization conditions for the O-3 oxidation reaction with 3 equiv. of Benzoquinone as oxidant.



Entry	Catalyst (mol%)	Solvent	Temp (°C)
1	2.5	ACN:H_2O (10:1)	r.t.
2[a]	2.5	ACN:H_2O (10:1)	r.t.
3[b]	2.5	ACN:H_2O (10:1)	r.t.
4[c]	6	DMSO:Dioxane (1:4)	r.t.

[a] reaction with TBDMSCl after the oxidation reaction; [b] protected from light; [c] gradual additions of 2 equiv. every two hours.

Despite all efforts to apply this methodology to these substrates, formation of the O-3 ketone was not detected.

2.2.3 Preliminary studies on the modification of the NAc group

Literature search was performed on the described procedures that allow replacement of the NAc group by another group useful to obtain the desired NAG-NAM product. At this point a procedure reported by Georg's group[56] was attempted. This procedure consists of using the Schwartz reagent to convert amides into the corresponding imines and then by a hydrozircornation of the heteroatom occurs leading to the amine and the corresponding aldehyde, Figure 2.23.

This route caught our attention since it could be applied to generate an extremely useful intermediate- **II.74**. Thus, by treating **II.53** with sodium methoxide in dry methanol the deacetylated product could be obtained in quantitative yield. Then once the imine **II.73** was formed a simple slightly acidic work-up followed by a phthaloylation reaction could deliver

II.74. This final product would be an ideal starting material to the desired synthesis, since it can be fully regioselectively modified using standard procedures.

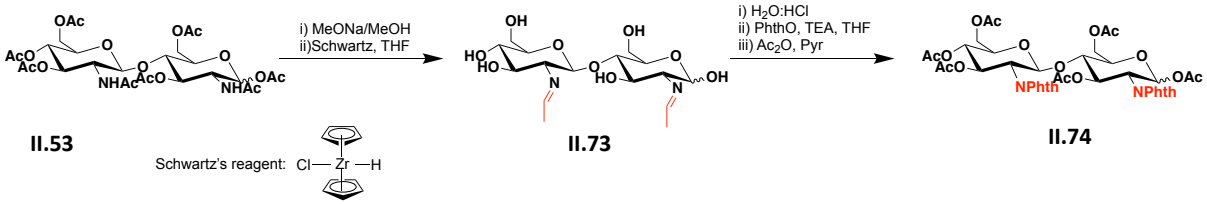
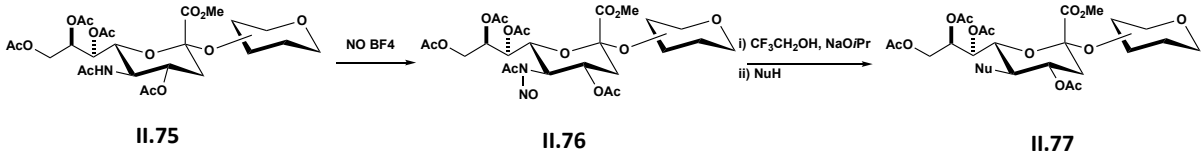


Figure 2.23 Synthetic plan to form NPhthchitobiose (II.74) using Schwartz's reagent.

The challenge of this synthetic route relied on the isolation of the product from hydrolysis of **II.53** since chitobiose is not easy to handle due to its hydrophilic character.

The synthesis started with methanolysis of **II.53**. Then 1.2 equiv. of Schwartz reagent for each NAc group was used. The Schwartz's reagent was added to a bottom round flask in inert atmosphere in order to prevent the presence of water. Then the product was dissolved in THF and added to the Schwartz solution and let to react overnight, since it was no evidence of the imine formation by TLC. After the overnight reaction was visible, by TLC with higher RF comparing with the starting material, a product presumed to be the imine product, **II.73**. Despite all efforts it was not possible to isolate **II.73** so the synthesis proceeded with the N-phthaloylation step using TEA to neutralize the hydrochloric salt formed by the acidic work-up and phthalic anhydride, in THF at reflux overnight. Again, the isolation process was not easy since the product had many free hydroxy groups. After ^{1}H-NMR analysis of the crude it was not clear if the phthaloyl group was installed. Thus, acetylation of the crude was performed with acetic anhydride in pyridine in order to clarify the spectra. However, **II.74** seemed to be just partially phthaloylated despite the use of a high loading of phthalic anhydride (3 equiv. per amine group). It seems that this reaction is highly dependent on the π electron system of the amide and with that in mind we figured out that our system was not the best to apply this methodology.[56]

In 2013, Crich and his co-worker reported this very interesting methodology[57] applied to a sialic acid disaccharide (**II.75**), in which the-N-acetyl groupwas converted into other interesting functional groups, such as azide, acetyl, alcohols and others, Figure 2.24.



II.75 **II.76** **II.77**

Figure 2.24 Conversion of the NHAc group into other functional groups by NAc-NO modification.

This Crich work[57] was adapted from a work published by Zbiral group[58] where this methodology was applied to neuramic acids derivatives. This methodology was firstly applied to the sialic acid monosaccharide where it proved to be very selective and useful.[59]

This strategy inspired us to understand whether it could be applied to the chitobiose derivatives.[33] This would allow to overcome the problems associated with the use of NAc as well as to install a wide range of functional groups at the position C-2 of the glucosamine unit.

II.53 **II.78** **II.79** **II.80**

Figure 2.25 Proposed conversion of N-peracetylated chitobiose into other functional groups using the methodology developed by Crich.[57]

Figure 2.25 shows the versatility of this strategy. Compound **II.53** is an easy to prepare material, and this methodology was never applied to this disaccharide. In fact, this strategy was rarely explored on disaccharides at all, even in monosaccharides. Once **II.78** is formed, a base mediated decarboxylation would occur to form **II.79**. If these previous steps were accomplished the difficult part would be the protonation to extrude N_2 with simultaneous nucleophilic attack to install the new functional group. Different nucleophiles could be tested in order to create a library of new or easy to prepare disaccharides.

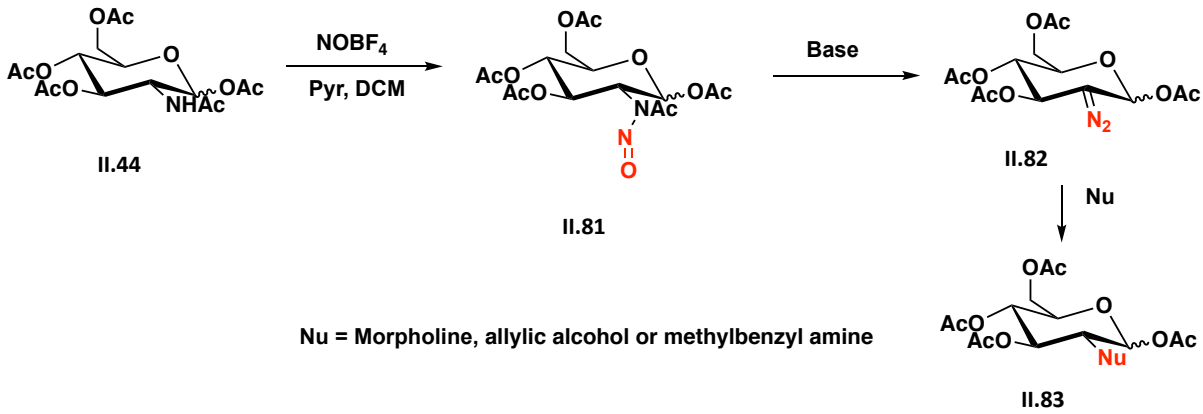


Figure 2.26 Conversion of NHAc into other functional groups using Crich methodology[57] applied on II.44.

Since the initial strategy aimed to replace the NAc group by other groups, morpholine, allylic alcohol and methyl benzyl amine were chosen as model nucleophiles. The choice of allylic alcohol is due to the propagylic alcohol reported by Crich .[57]

Crich[57], reported the conversion of the NAc group into, propargylic ether, fluoride, isopropilic ether, OAc and SAc. It was a good foundation for use to try different groups in a different disaccharide. Using a phtalimide salt as nucleophile, it would solve one of the major bottlenecks of PGN fragments synthesis.

First attempts were performed with both disaccharide **II.53**, Figure 2.25, and monosaccharide **II.44**, Figure 2.26, as starting materials to tune the reaction conditions.

The procedure to synthesize compound **II.81** was the same as described.[59] The **II.81** is thermally unstable so it had to be freshly synthesized before each reaction, using 4 equiv. of $NOBF_4$, 10 equiv. of pyridine in DCM in 0.1M of **II.44**, at 0 °C. The reaction was stirred for 6 hours. The reaction mixture was turning fluorescent yellow with the increase of reaction time up to 6 hours. The work-up was done by washing with cold HCl 1M, cold saturated $NaHCO_3$ and cold brine. Then the solvents were removed under reduce pressure always keeping the crude at 0 °C. The product **II.81** was obtained but revealed to be unstable and was used without further purification.

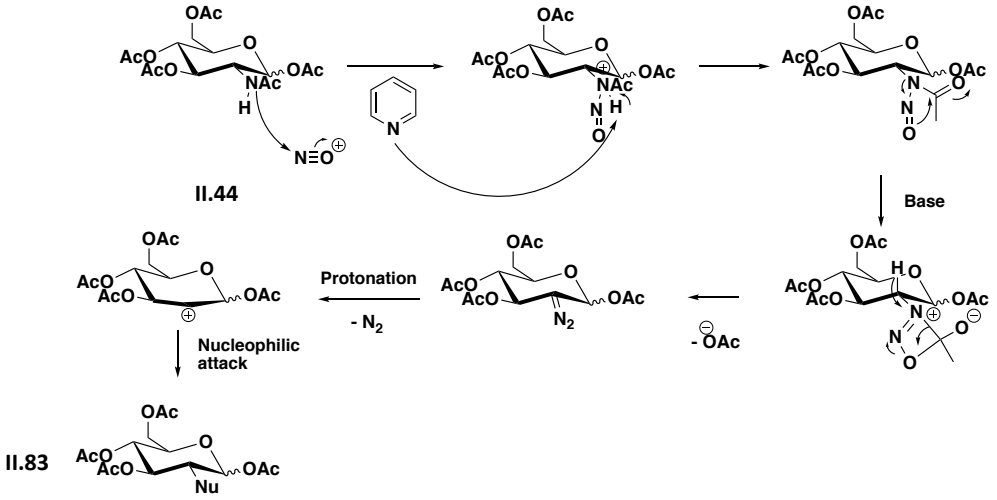


Figure 2.27 Proposed reaction mechanism to convert II.44 into II.83.

Figure 2.27 elucidates the reaction mechanism for formation of **II.83**.

It was soon anticipated that this methodology when applied to glucosamine could generate glucose pentaacetate (**II.84**) as product, Figure 2.28. Thus, various trials were taken in order to understand how could the nucleophilic attack be favored and avoid the formation of the glucose pentaacetate (**II.84**).

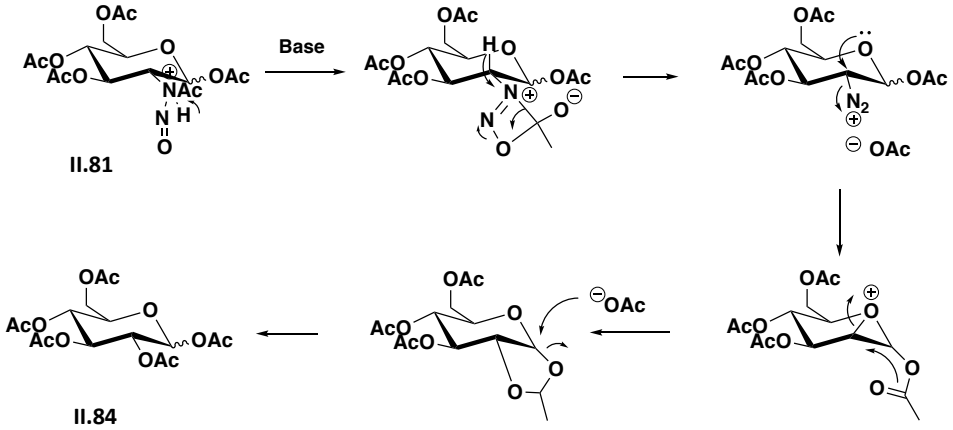
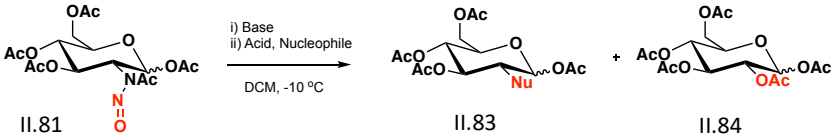


Figure 2.28 Reaction mechanism to afford glucose pentaacetate II.84 from II.81.[60]

Table 2.4 summarizes all the trials made with the monosaccharide **II.44** as starting material, for the replacement of NAc by other N-protecting group.

The entry 1 was carried using the standard procedure[57], where methylbenzyl amine was used since it has aromatic protons and it would be easily detected. However due to the high amount of this reagent used, the isolation of the product was difficult as well as the monitoring of the reaction. So, the nucleophile loading was reduced to 10 equiv., entry 2, affording **II.84** in 7% yield.

Table 2.4 Screening of the reaction conditions for preparation of II:83 from II.81.



II.81 II.83 II.84

Entry	Base system (equiv.)	Acid (equiv.)	Nucleophile (equiv.)	Yield **II.83** ; **II.84** (%)
1	Trifluoro ethanol/ Sodium isopropoxide (1.2)	-	Methyl benzyl amine (20)	n.d
2	Trifluoro ethanol/ Sodium isopropoxide (1.2)	-	Morpholine (10)	0 ; 7
3	Trifluoro ethanol/ Sodium isopropoxide (1.2)	-	Morpholine (1.5)	0 ; 11
4	Trifluoroethoxide / 18-crown-6 (2)	-	Morpholine (1.0)	n.d
5	Trifluoroethoxide / 18-crown-6 (2)	*p*TsOH (0.1)	Morpholine (1.1)	n.d
6	Trifluoroethoxide / 18-crown-6 (2)	HBF_4 (0.5)	Morpholine (1.1)	0 ; 3.3
7	Trifluoroethoxide / 18-crown-6 (2)	HBF_4 (0.5)	-	n.d
8	Trifluoroethoxide / 18-crown-6 (2)	HBF_4 (0.5)	Allylic alcohol (20)	0 ; 15
9	Trifluoroethoxide / 18-crown-6 (2)	HBF_4 (0.5)	Allylic alcohol (1.1)	0 ; 27
10	Trifluoroethoxide / 18-crown-6 (2)	HBF_4 (0.5)	Morpholine (20)	0 ; 18

n.d. nothing detected

Once again, the high amount difficulted the reaction monitoring, and the loading was decreased to 1.5, entry 3, affording **II.84** in 11% yield. Morpholine was not detected in the ^{1}H-NMR spectra. The procedure was changed, entry 4, and instead of a mixture of trifluoroethanol and sodium isopropoxide, a mixture trifluoroethoxide and a crown ether (18-crown-6) was used as reported.[57] At this point it was considered that the morpholine could react with HBF_4, preventing the protonation of **II.82**. According to the observations, the problem seemed not to be the base component of the reaction but the acid addictive that was not present at the time. When *p*TsOH was used, entry 5, no product was observed. Next, HBF_4 was used as proton source,since it is an acid which the corresponding conjugate base can not act as a nucleophile. Entry 6 and 7 were performed in parallel, entry 7 as a control of

entry 6 where **II.84** was isolated in 3.3% yield. Both experiments gave the same ^{1}H-NMR spectra, using morpholine as nucleophile, that could also neutralize all the HBF_4 and would not be able to protonate **II.82**. Thus, on entry 8 the nucleophile was changed to the allylic alcohol. However, the high amount made it difficult to isolate the product affording **II.84** in 15% yield. Allylic alcohol amount was reduced to 1.1 equiv. (entry 9) and **II.84** was isolated in 27% yield, confirmed by ^{1}H-NMR. Last trial (entry 10) was performed to ensure that the HBF_4 reacted with the **II.82** before adding the morpholine. The product was isolated after flash chromatography and confirmed to be **II.84** in 18% yield, confirmed by N(H)HSQC spectra, Figure 2.29.

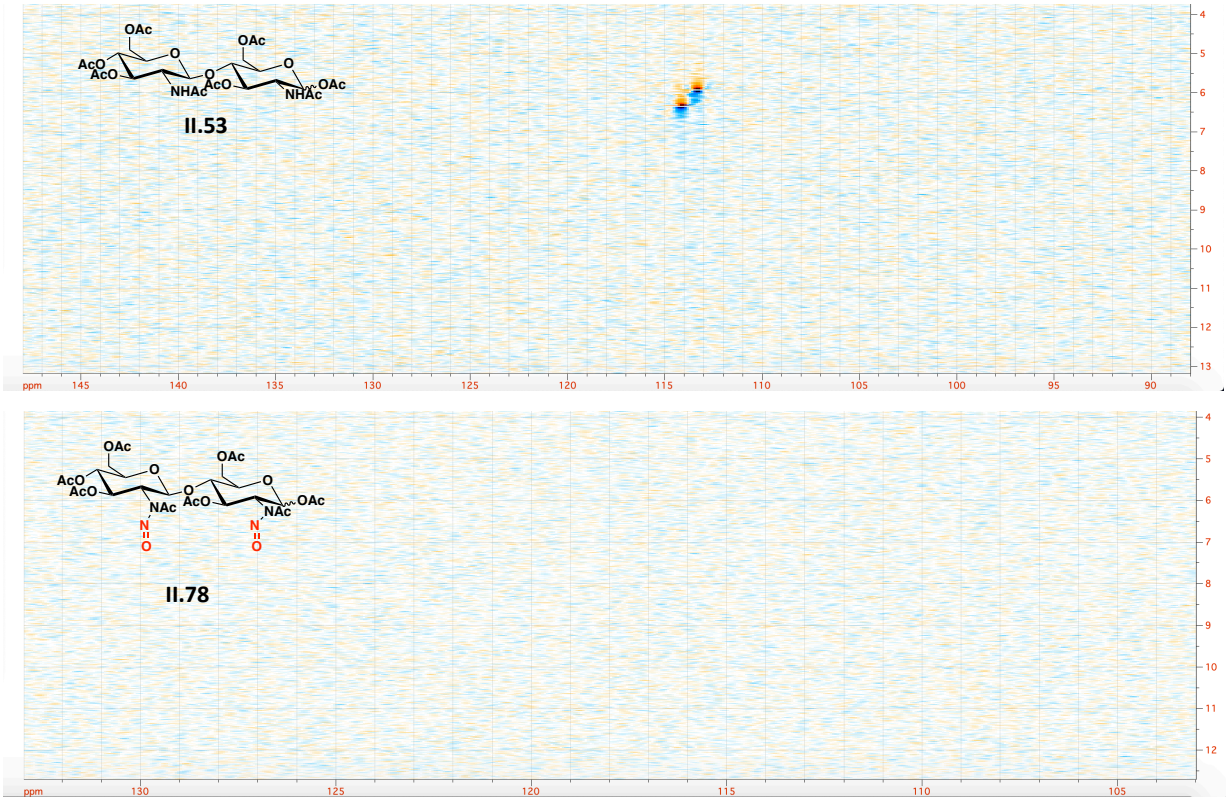


Figure 2.29 N(H)HSQC experiment of II.53 and II.78. $CDCl_3$, 278K, f1 (88.9 – 148 ppm), f2 (-3.7 – 13.18 ppm).

It is not clear if, the acetate group which is eliminated is responsible for the nucleophilic attack. If acetic acid is formed, it can protonate the **II.82** intermediate and then the acetate group could attack the carbocation to form the glucose pentaacetate. However, in 1973 Williams group reported a thermal decomposition of the compound **II.81** to glucose pentaacetate, Figure 2.27.[60] The mechanism reported postulates the participation of the oxygen from the sugar ring to extrude N_2 giving the bicyclic compound. Then the acetyl group

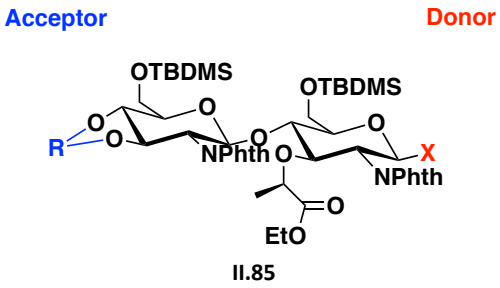
from the anomeric position, forms the acetoxonium ion which undergoes a nucleophilic attack from the acetate group, Figure 2.28.

Nevertheless, the reaction was performed with the disaccharide to investigate whether the formation of the **II.78** occurred as expected. In fact, by N(H)HSQC experiments we were able to confirm the disappearance of the both N-H signals, Figure 2.29.

This strategy allowed the transformation of the peracetylated glucosamine (**II.44**) into peracetylated glucose (**II.84**). The aim of converting peracetylated glucosamine (**II.44**) into other functionalized sugar varying the nucleophile was not accomplished.

2.2.4 Approach towards NAG-NAM disaccharidevia a di-NPhthchitobiose derivative

In this study, we came across with several difficulties, mainly due to the effect of the substituents at the amino moiety. Thus, it was envisaged to synthesize a molecule similar to **II.85**, Figure 2.30, that could work as both donor and acceptor.



II.85

Figure 2.30 Versatile fully functionalized molecule that could be used as both acceptor and donor.

In order to be able to create the NAG-NAM precursor **II.85**, Figure 2.30, NAc moiety in **II.53** had to be modified into another group. Thus, a synthetic route was designed according to Figure 2.31.

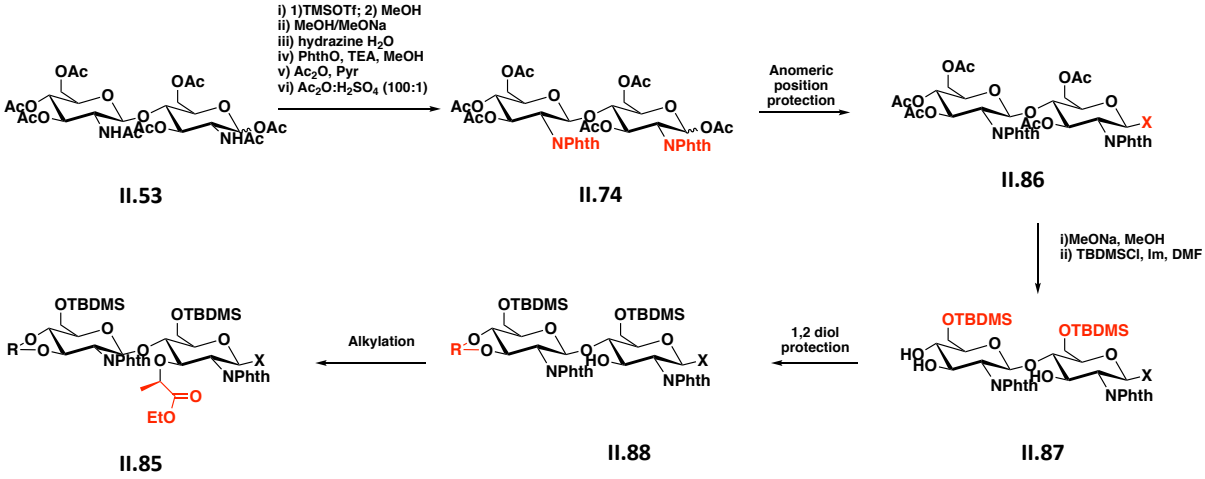


Figure 2.31 Synthetic route designed to synthesize II.85.

By converting the N-Acetyl group in **II.53** into the N-phthaloyl **II.74**, the absence of hydrogen atoms linked to the amine, would not only increase the solubility in organic solvents, but also increase the nucleophilicity, of the acceptor moiety, by avoiding the formation of a hydrogen bond. Then, for the anomeric position group (-X), **II.86**, several groups could be tested such as, OTBDMS, OTBDPS, STol, OAllyl. The advantage of STol group in comparison with other is the possibility of being selectively activated to preform glycosylation reactions. Methanolysis of **II.86** followed by a sylilation with TBDMSCl would allow to access **II.87**. Simultaneous protection of the O-4 O-3, 1,2 diol, to form **II.88**, could be carried using phosgene, CDI or a dione.[61] Additionally, as reported by Walker[17] once a O-3 and O-4 are free, the glycosylation undergoes through the O-4 hydroxy group. A final step of lactyl insertion could be performed using the same reaction conditions as used to produce **II.85**. We anticipated a higher yield in this last synthetic step since the NHAc would not be present.

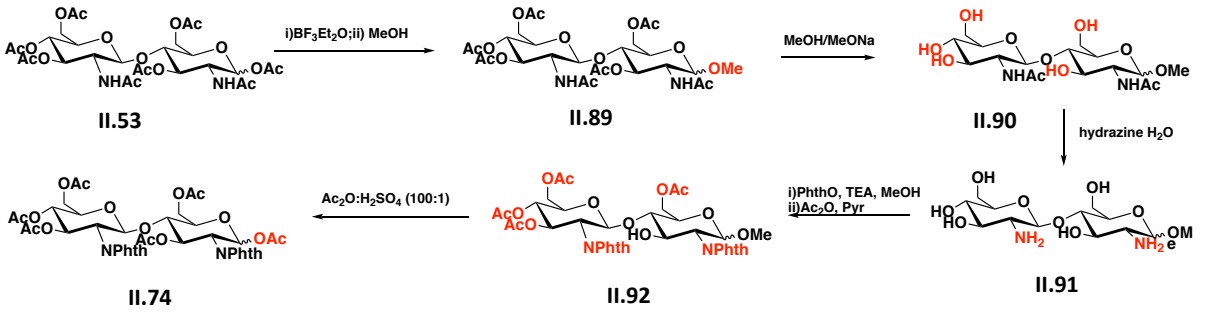


Figure 2.32 Synthetic route designed to convert NHAc into NPhth peracetylated chitobiose.

In order to obtain NPhth derivative **II.74**, we designed a five steps synthetic plan shown in Figure 2.32. Although it might be a straightforward synthetic route, the key step, is the formation of **II.74**. Thus, the synthesis started with preparation of **II.74** by applying a protocol reported by Hui group to prepare this compound, Figure 2.32.[62] We found out that the reaction of **II.53** with methanol was more efficient if $BF_3.Et_2O$ was used as promotor, rather than TMSOTf (by TLC monitoring of consumption of the starting material). Compound **II.53** is a mixture of α and β isomers and the authors reported the reaction using only the β-anomer. Nevertheless, the formation of **II.89** was confirmed by ^{1}H-NMR. The following steps were inspired in Kuzuhara reported work.[63] Kuzuhara reported the conversion of **II.89** into **II.74**, Figure 2.31. Accordingly, a methanolysis reaction of **II.89** using sodium methoxide in dry methanol overnight was performed in order to obtain **II.90** in a quantitative yield. Next, the acetyl groups were removed from **II.90** with hydrazine. The reaction was carried under reflux conditions for 24 hours, followed by the N-phthaloylation reaction and O-acetylation **II.92** after an acetylation step. **II.92** was isolated, after column chromatography, in a 34% yield, confirmed by ^{1}H-NMR, from the initial **II.53**.

The final step consisted in the replacement of the methoxyl group at the anomeric position by O-acetyl, **II.74**, in order to be then converted into the -X group, **II.86**, as initially designed, Figure 2.30. The final reaction to obtain the **II.74** was already reported.[63] This step was described as an acetolysis (100:1 Ac_2O:H_2SO_4) at room temperature of the OMe group followed by an acetylation of the hydroxy group at the anomeric position. However, this reaction did not occur as predicted and the product **II.74** was not isolated. By ^{1}H-NMR spectra analysis the product was not present. On the basis of the obtained results, an alternative route was considered, since a more suitable group at the anomeric position, rather than the methoxy group, could be used to achieve a versatile molecule such as **II.85**.

2.2.5 Approach towards NAG-NAM disaccharide via the di-N-TFA chitobiose derivative

The next approach consisted on the preparation of per-N-trifluoroacetyl derivatives via a direct and chemoselective *trans*-N-acylation of peracetylated COSs, as a suitable starting material to achieve the NAG-NAM precursor.

The synthesis started with the controlled depolymerization of chitin by acetolysis,[64, 65] to obtain the corresponding per-acetylated di-, tri- and tetrasaccharides, using an optimized method described by Beau group, Figure 2.33.[33]

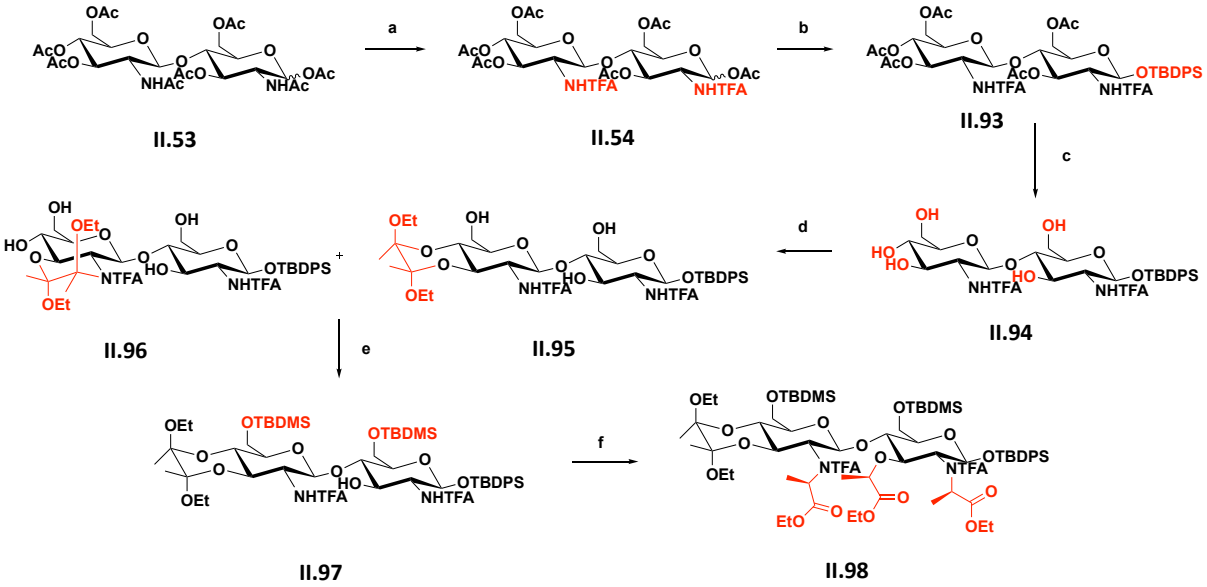


Figure 2.33 First approach towards NAG-NAM precursor synthesis. a) TFAA, Pyr, 120 °C, 15 min, 76%.; b) i) NH_2NH_2AcOH, DMF, 55 °C; ii) TBDMSCl, imidazole, DMF, 81%; c) MeONa, MeOH, r.t., 100%; d) butane-2,3-dione, *p*-TsOH, triethylorthoformate, EtOH, 60 °C, 24 h, 70%; e) TBDMSCl, TEA, cat. DMAP, DMF, 38%; f) i) NaH, DCM, 30 min.; ii) ethyl (*S*)-(–)2-(trifluoromethylsulfonyloxy)propionate, 38%.

In order to overcome the low solubility of N-peracetylated chitobiose **II.53**, a chemoselective *trans*-N-acylation was performed to convert **II.53** into the N-trifluoroacetyl derivative (NHTFA) **II.54**,[33] by reaction of **II.53** with trifluoroacetyl anhydride in pyridine. The mechanism of this transformation is depicted in Figure 2.34. The N-TFA group can be either easily replaced by the N-acetyl group in a more advanced stage of the synthesis or provide an efficient donor in a future glycosylation reaction. Opposite to the N-acetyl glycosides, the N-TFA derivatives possess a higher solubility in common organic solvents. Having the N-trifluoroacetylated intermediate **II.54** in hands, the next step consisted on the choice of a proper anomeric protecting group, which is critical and should withstand the reaction conditions of different steps that we expected to apply, while being easily removed in a late stage of the synthesis.

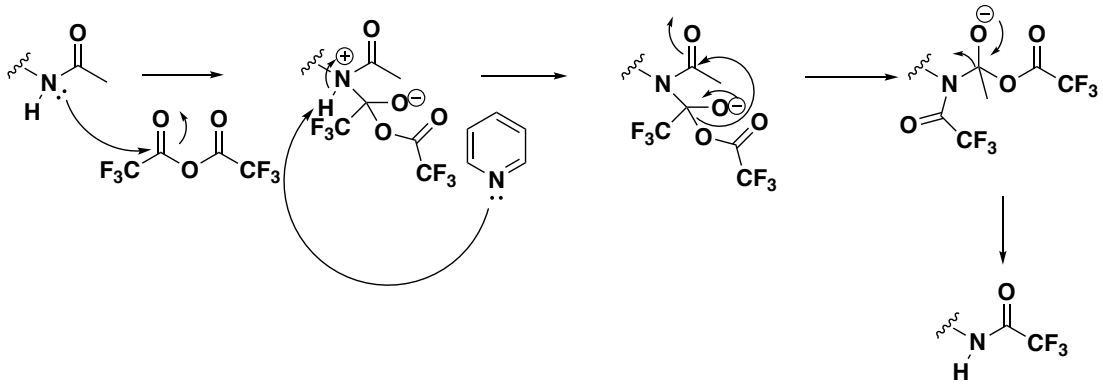


Figure 2.34 Mechanism of transacylation reaction of the N-acetyl goup to give the N-trifluoroacetyl group.

Silyl ethers can be employed as temporary protecting groups of the anomeric position, and these intermediates can give rise to a variety of glycosyl donors.[15, 64, 65] TBDPSCl was used to protect anomeric position using imidazole in DMF for 5 hours at room temperature, Figure 2.33. Thus compound **II.93** was isolated in 81% yield and the acetyl groups removed to afford **II.59** in quantitative yield.

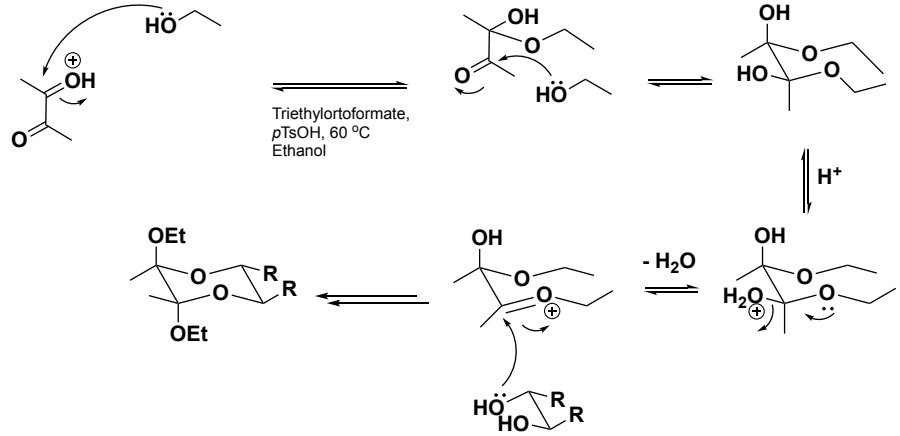


Figure 2.35 Mechanism of the protection of the hydroxy groups using butane-2,2-dione.

Next, the protection of O-4 and O-3 in **II.94** was performed by treatment with butane-2,3-dione, and removal of the silyl groups was not observed. The mechanism for this transformation is depicted in Figure 2.35. However, a mixture of two products could be detected by NMR which was identified in the ^{1}H NMR spectra by the appearance of a triplet at 4.25 ppm, with 10.6 Hz *J* constant and the multiplete modification at 4.03 – 3.34 corresponding to the C-3 and C-4 and C-2, in a 1:0.3 (C-4:C-2) ratio, corresponding to the compounds **II.95** and **II.96**, respectively.

Compound **II.95** was formed in 70% yield, along with the undesired side-product that contained the same features, such as TBDPS group and the diacetal moiety, although with a

different NMR profile, which was assigned to structure **II.97**. This lack of selectivity was previously observed[66, 67] for glucose, although not yet reported for glucosamine derivatives.[68] Unfortunately, both compounds possessed a very similar retention time in the conditions used, which hampered its chromatographic separation, and consequently its full characterization. This mixture was then used directly in the next step and the silylated compound **II.97** was isolated in 38% after chromatographic purification.

The subsequent step consisted on the lactate insertion using the propionic ester triflate. By treatment of **II.97** with sodium hydride in dry DCM at 0 ^{o}C for 30 minutes, the deprotonated intermediate was obtained. Next, the (*S*)-2-propionic ester triflate was added and the reaction stirred for 6 hours. The crude reaction mixture revealed the formation of several products, that by ^{1}H NMR analysis correspond to insertion of the lactyl moiety at the O-3 as well as at the N-TFA groups. This conclusion arises from the ^{1}H-NMR analysis of compound **II.98**, where the two Hα corresponding to the N-lactyl moiety are observed at 5.3 ppm while the O-lactyl appears at 4.7 ppm, also three ethyl moieties are found, 1.40 – 1.14 ppm and 1.03 – 0.79 ppm. The strong electron-withdrawing effect provided by the trifluoroacetyl group, increases the acidity of the NHTFA, that can be easily deprotonated in the presence of sodium hydride to attain the N-lactyl products. Compound **II.98** was identified as the major product, isolated in 38% yield. Consequently, we considered to perform the deprotection of trifluoroacetyl group followed by N-acetylation prior to the lactate insertion. Several attempts were made to attain the N-acetylated compound from **II.98**: 1) LiOH in H_2O:MeOH and Ac_2O/K_2CO_3; 2) Zn- AcOH in EtOAc; 3) LiOH in dry THF and 4) $NaBH_4$ in dry ethanol followed by Ac_2O. Despite all the efforts, none of the conditions was efficient due to the presence of the TBDPS group, since the use of LiOH promotes the silyl group removal by hydrolysis[33] at the anomeric position.

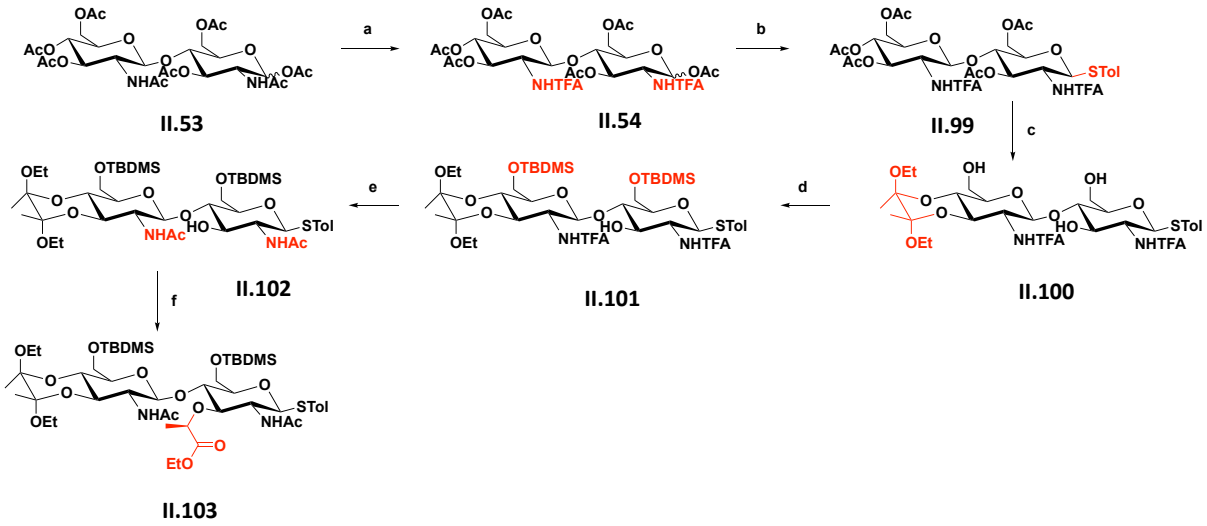


Figure 2.36 Second approach towards NAG-NAM precursor a) TFAA, Pyr, 120 °C, 15 min, 76%; b) TolSH, BF_3OEt_2, DCM, r.t., overnight, 80%; c) i) MeONa, MeOH, r.t., overnight. ii) butane-2,3-dione, *p*-TsOH, triethylorthoformate, EtOH, 60 °C, 16 h, 58%; d) TBDMSCl, DMAP, TEA, DCM, 56%. e) i) NaOH 2 M, THF, 80 °C, 3 h, ii) Ac_2O, pyridine, DCM, r.t., overnight, 56%; f) i). NaH, DCM, 30 min; ii) Ethyl (*S*)-2-(trifluoromethylsulfonyloxy)propionate, r.t., 16 h, 38%.

Given the results reported above, it was then envisaged to use STolyl (STol) group at the anomeric position. This group was installed using *p*-methylthiophenol under $BF_3.OEt_2$ conditions to obtain **II.99**, in 80% yield, Figure 2.36. Following the previously devised synthetic plan, methanolysis of **II.99** and successive protection of 3,4-O positions with the butane-2,3-diacetal moiety afforded compound **II.100** in 58% yield. Once again as in the previous synthesis the reaction led to a by-product, N,O-ring formation, but we were able to separate them through silica chromatography. Subsequent protection of both O-6 with TBDMS afforded **II.101**. By treatment of **II.100** with TBDMSCl TEA and DMAP in DMF for 16 hours afforded **II.101** in 56% yield. To avoid the random introduction of the lactate moiety, the NTFA group was replaced by NAc group. This step was carried by treatment of **II.102** with NaOH in THF at 80 °C, followed by N-acetylation with acetic anhydride in pyridine, affording **II.102**, confirmed by ^{1}H-NMR by the appearance of acetyl signals, with a 56% yield. This intermediate **II.102** possesses an O-3 free that allows the selective introduction of the lactyl moiety. Thus, the lactyl unit was introduced by treatment of **II.102** with propionic ester triflate, achieving **II.103** in 38% yield, confirmed by ^{1}H-NMR by the appearance of, doublet at 4.67 ppm and a triplet at 1.27, both corresponding to the lactyl unit. Also, 2D NMR experiments as COSY and HBMC confirmed the correct introduction at the O-3 position, HRMS also gave the exact mass of the compound **II.103**.

This compound possesses all functional groups (O-lactyl and NAc) required to prepare the NAG-NAM disaccharide, constituting an excellent precursor of this moiety. An alternative anomeric protecting group was further investigated, the 2-methyl-5-*tert*-butylthiophenol. However, no improvement of the overall synthetic scheme was achieved. This is mainly due to the difficulties in the sylilation of O-6 positions with TBDMSCl, that revealed to be cumbersome, indeed a complex mixture was observed, maybe as a consequence of a higher steric hindrance. Despite its odor HSTol revealed to be a superior protecting group for this synthesis. Compound **II.103** represents an advanced precursor of the NAG-NAM disaccharide, since the remaining protection groups can be removed by applying well-established procedures.[61] In this sense, this synthesis constitutes a semisynthesis of a NAG-NAM containing moiety, a challenging disaccharide, from naturally abundant chitobiose.

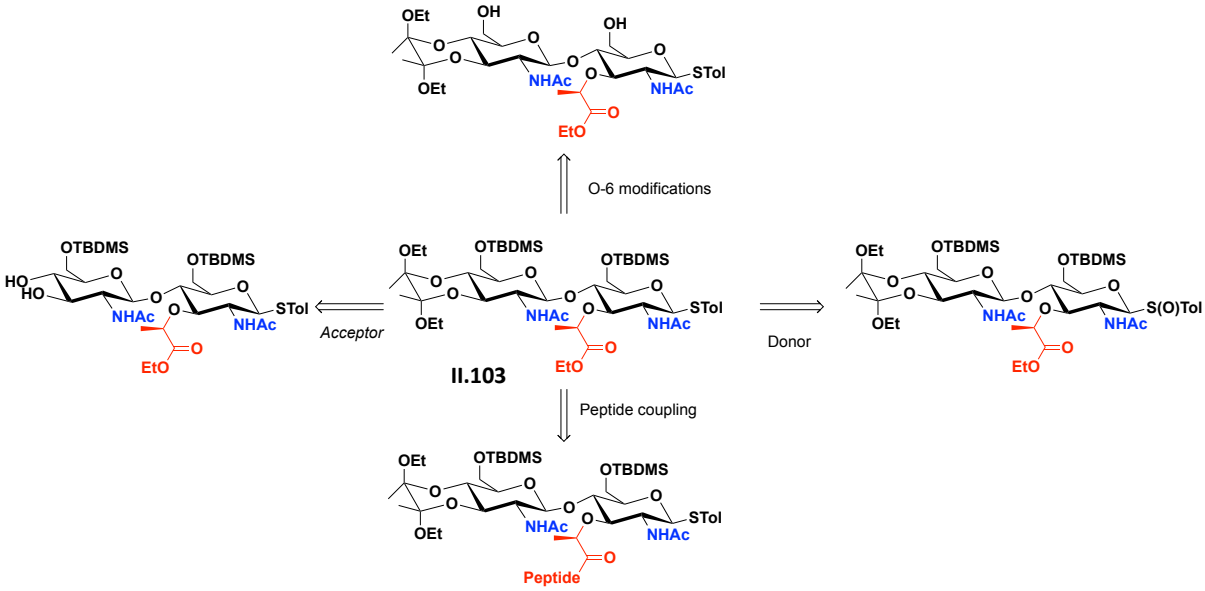


Figure 2.37 Versatility of the NAG-NAM precursor II.103 and its wide applications.

This protocol takes advantage of the already installed β-(1,4) glycosydic bond, present in chitobiose, which constitutes the main bottleneck of traditional approaches. This simple approach provides a fast access to a versatile intermediate **II.103**, from a gram-scale peracetylated disaccharide (**II.53**). In addition, **II.103** allows further regioselective manipulation, by selective removal of the protecting groups, Figure 2.37. After STol oxidation with NIS and deprotection of butane-2,3-dione the **II.103** would be allowed to self-condensate.

The protecting groups removal is reported in literature.[2, 24, 69] Despite our efforts, we could not completely remove the butane-2,3-dione protecting group, using 5-20%TFA, *p*TsOH 5%. The group seemed to be partially removed (^{1}H-NMR confirmation). More acidic conditions could jeopardize the glycosidic bond itself. Despite the difficulties associated with 2-acetamido glucosamine acceptors and donors, this intermediate might also provide size-defined oligosaccharides, as well as glycopeptides, highly useful for biological investigations.

2.3 Conclusions

This chapter reports the synthetic studies developed towards the synthesis of a versatile precursor of the NAG-NAM disaccharide.

Different strategies were attempted: i) regioselective oxidation of the O-3 position from the terminal residue of a NHTFA chitobiose derivative , by applying a preformed palladium-catalyst reported by Minnaard group; ii) removal of the N-acetyl group from N-acetylglucosamine by using either the Schwartz reagent, through its imine formation and subsequent protection; or using the methodology developed by the Crich group for sialic acid, where the NAc group is converted in NAcNO and then replaced by a nucleophile; iii) reduction of the NHAc group from N-Acetyl chitobiose with hydrazine followed by protection with phthalimide group.

Despite of all attempts, either with glucosamine derivatives or chitobiose derivatives, the desired products were not observed.

The last approach started with preparation of peracetylated chitobiose (**II.53**), obtained in a gram scale from acetolysis of chitin. We took advantage of the already established β-1-4 glycosidic bond, present in the peracetylated chitobiose, and avoided one of the most challenging steps in the synthesis of peptidoglycan fragments: the regio- and enantioselective glycosylation reaction. This complex carbohydrate

synthesis, NAG-NAM oligosaccharides, commonly involves multistep sequences to install the lactyl moiety at O-3 and control the enantioselective glycosylation. This new strategy relied on the different protection of the hydroxy groups, using NTFA, STol, diacetal and silyl protecting groups, and allowed a regioselective introduction of the critical lactyl unit of a versatile intermediate (**II.103**).

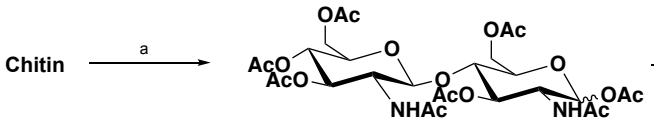
The overall strategy resulted in a considerable reduction of the number of steps (5 steps from **II.53**) required to a NAG-NAM containing disaccharide, constituting an atom-economy procedure. This unprecedented route provides a new platform for the preparation of bacterial peptidoglycan and related non-natural derivates.

2.4 Experimental

All commercially obtained reagents were used without further purification unless specified. All the mentioned solvents used in the reactions were dried by usual methods. MS 4Å were activated by heating at 300 ^{o}C for 3 h. Preparative and analytical TLC was performed with silica gel 60 plates of 1 mm, 0.5 mm and 0.25 mm, respectively. Nuclear Magnetic Resonance (NMR) spectra were recorded at Bruker Advance 400 MHz for ^{1}H and at 100 MHz for ^{13}C, in $CDCl_3$, DMSO-d_6 or D_2O with chemical shift values (δ) in ppm downfield from TMS (0 ppm) or the solvent residual peak of D_2O (4.79 ppm), DMSO-d_6 (2.50 ppm) or $CDCl_3$ (7.24 ppm) as internal standard. The chemical shifts (δ) for proton spectra were expressed in parts per million (ppm) and the data obtained was presented in the following order: deuterated solvent, signal chemical shift (δ), relative intensity, spin multiplicity (s –singlet, d – doublet, t – triplet, m – multiplet, dd – doublet of duplets), coupling constant (J, in Hz) and molecule peak attribution if possible. The data for carbon spectra was presented in the following order: solvent, chemical shift (δ), molecule attribution if possible. Infrared (IR) spectra were recorded on a Bruker Tensor 27 spectrophotometer FTIR spectra were recorded on Perkin-Elmer Spectrum 1000 model apparatus in KBr dispersions for solid samples or NaCl dispersions for oil samples. In each spectra description only the more intense and characteristic bands were identified. The data obtained is presented in the following order: sample support (NaCl or KBr); frequency of the maximum absorption band (ν_{max} in cm^{-1}attribution to a functional group in a molecule if possible.

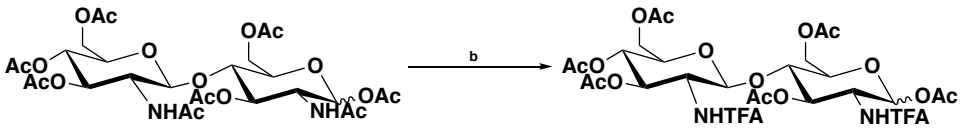
The reactions were followed by thin layer chromatography (TLC) silica gel 60 G/UV254 Macherey-Nagel with 0.20 mm. Spots detection on TLC was carried with UV light using a 254 nm lamp (Vilber-Lourmat). Additionally, TLC plates visualization was carried with a TLC spray solution of ethanol-sulfuric acid 9:1.

2.4.1 Preliminary synthesis



II.53

Synthesis of peracetylated disaccharide (II.53). Chitin (60.0 g) was added portion-wise to an ice-cold mixture of acetic anhydride (300 mL) and sulfuric acid (30 mL). The suspension was stirred at 55 °C for 3 h to give a brown mixture, kept at room temperature for 16 h and then heated at 55 °C for 1 h to give a homogenous solution. This mixture was then poured into an ice-cold solution of sodium acetate (120 g) in water (1.2 L). The resulting solid was filtered off. The filtrate was extracted with dichloromethane (3 x 1.5 L). The combined organic layers were successively washed with water (4.5 L), an ice-cold saturated aqueous $NaHCO_3$ solution (4.5 L), and water (4.5 L). The organic layer was dried over Na_2SO_4, and the solvent was removed under reduced pressure to afford a yellow solid (46.7 g). The residue was purified by silica gel chromatography (DCM/acetone from 9/1 to 1/9) to give the peracetylated disaccharide (**II.53**) as a white foam (9 g). ^{1}H NMR (400 MHz, $CDCl_3$) δ 6.09 (d, *J* = 3.5 Hz, 1H), 6.04 (d, *J* = 9.3 Hz, 1H), 5.69 (d, *J* = 9.1 Hz, 1H), 5.21 (dd, *J* = 10.9, 9.2 Hz, 1H), 5.16 – 5.09 (m, 1H), 5.04 (dd, *J* = 9.6 Hz, 1H), 4.49 – 4.32 (m, 3H), 4.23 – 3.88 (m, 5H), 3.73 (dd, *J* = 9.5 Hz, 1H), 3.62 (m, 1H), 2.18 (s, 3H), 2.14 (s, 3H), 2.07 (s, 3H), 2.05 (s, 3H), 2.00 (s, 3H), 2.00 (s, 3H), 1.94 (s, 3H), 1.92 (s, 3H). The data obtained is in accordance with the literature.[33]



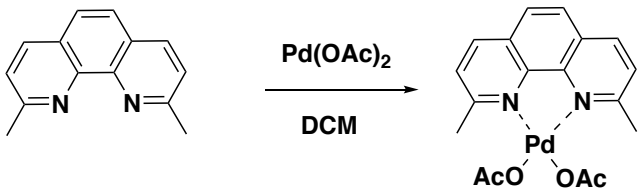
II.53 **II.54**

Synthesis of II.54. To a stirred 0.3 M solution of peracetylated disaccharide (**II.53**) (2 g) in dry pyridine (10 mL) was added trifluoroacetic anhydride (3.4 mL, 4 equiv. per NHAc). The mixture was heated at 135 °C in a sealed tube and stirred for 15 min. The mixture was then allowed to cool to room temperature and dry methanol was added. The resulting solution was stirred for additional 20 min. Solvents were removed under reduced pressure and the residue was dissolved in DCM. The organic solution was washed with an aqueous1 M HCl solution. The aqueous layer was extracted twice with DCM. The combined organic layers were washed with

brine, dried over Na_2SO_4, filtered and concentrated in vacuo. The residue was purified by flash-column chromatography on silica gel (silica, hexane/ethyl acetate, gradient 3/1 to 1/6) to afford **II.54** as a white solid (1.75 g, 76%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.46 (d, *J* = 9.4 Hz, 1H), 6.67 (d, *J* = 8.8 Hz, 1H), 6.19 (d, *J* = 3.5 Hz, 1H), 5.31 – 5.26 (m, 1H), 5.12 (dt, *J* = 22.2, 9.4 Hz, 2H), 4.53 – 4.31 (m, 4H), 4.09 (m, 3H), 3.89 (d, *J* = 10.3 Hz, 1H), 3.74 (t, *J* = 9.6 Hz, 1H), 3.66 (d, *J* = 8.1 Hz, 1H), 2.21 (s, 3H), 2.17 (s, 3H), 2.09 (s, 3H), 2.07 (s, 3H), 2.02 (s, 3H), 2.01 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 171.8, 171.7, 170.8, 170.5, 169.3, 168.7, 157.6, 101.0, 89.5, 75.5, 72.5, 72.0, 71.1, 70.0, 67.7, 61.6, 61.2, 54.6, 52.0, 21.0, 20.9, 20.7, 20.6, 20.5. The data obtained is in accordance with the literature.[33]

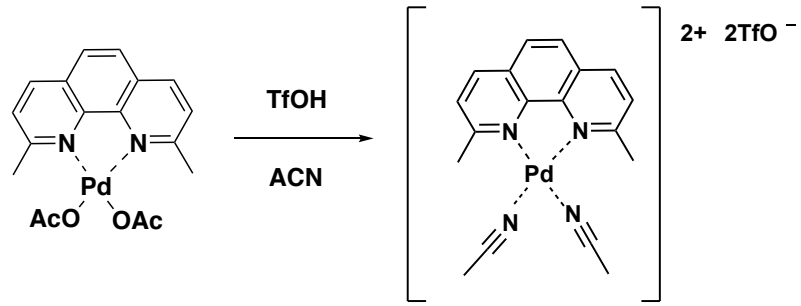
2.4.2 O-3 regioselective modification on chitobiose derivative

2.4.2.1 Preparation of the palladium catalyst



II.66 **II.67**

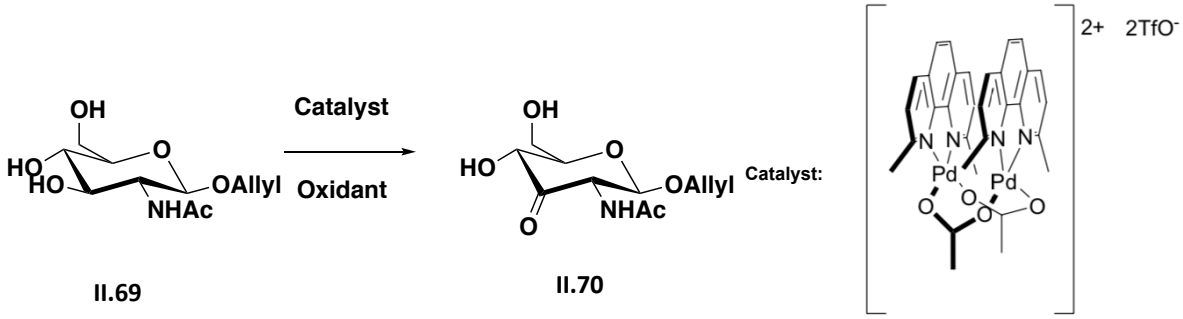
Synthesis of II.67. A solution of Neocuproine (**II.66**) (1.89 mmol, 400 mg) in anhydrous DCM (7 mL) was added to a solution of $Pd(OAc)_2$ (1.72 mmol, 385 mg) in anhydrous toluene (35 mL) at room temperature under nitrogen. The mixture was stirred overnight, and pentane was added to precipitate the complex. Solids were filtered off, washed with acetone and dried under vacuum to give **II.67** as a dark yellow solid (130 mg, 0.29 mmol) in 17% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.32 (d, *J* = 8.4 Hz, 2 H), 7.84 (s, 2 H), 7.47 (d, *J* = 8.4 Hz, 2 H), 2.92 (s, 6H), 2.04 (s, 6H). The data obtained is in accordance with the literature.[53]



II.67 **II.68**

Synthesis of II.68. To a slurry of **II.67** (0.13 mmol, 60 mg) in anhydrous acetonitrile (0.3 mL) was added a solution of triflic acid (0.338 mmol, 30 µL) in anhydrous acetonitrile (0.33 M, 1 mL) at room temperature under nitrogen. The mixture was stirred for 1 h and diethyl ether was added to precipitate the complex. Solids were filtered off and dried under vacuum to give **II.68** as a light yellow solid (81 mg, 0.115 mmol) in 88% yield. ^{1}H NMR (400 MHz, CD_3CN) δ 8.69 (d, *J* = 8.4 Hz, 2 H), 8.08 (s, 2 H), 7.78 (d, *J* = 8.4 Hz, 2 H), 2.99 (s, 6H), 1.96 (s, 6H). The data obtained is in accordance with the literature.[53]

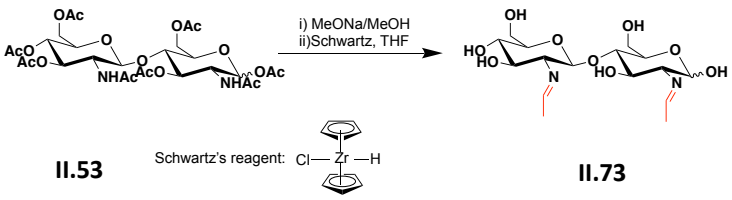
2.4.2.2 Oxidation experiments carried with the palladium catalyst



II.69 **II.70**

General procedure to oxidize the C3 position. To a bottom round flask with magnetic stirrer were added **II.67** (1.7 mg, 0.00235 mmol), **II.68** (0.994 mg, 0.00235 mmol), ACN (0.3 M, 3 mL) and H_2O (0.3 mL). The mixture was vigorously stirred at room temperature until the Pd complexes had dissolved completely. Then the **II.69** or **II.71**, (0.094 mmol), and benzoquinone (0.292 mmol, 30.5 mg) were added. The reaction was let to stirred overnight. The reaction was monitored by TLC using DCM:Acetone:MeOH:H_2O (56:20:20:4) as eluent. Toluene (5 mL) was added and the mixture was extracted twice with water (1 mL). The combined water layers were washed once with ethyl ether (5 mL), filtered and concentrated *in vacuo.* A colorless oil was obtained, however the ^{13}C-NMR did not reveal any signals for the desired product.

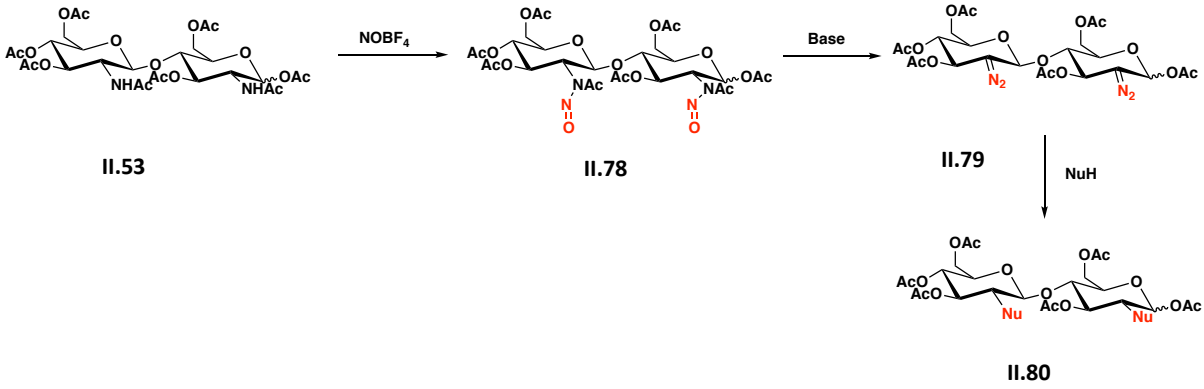
2.4.3 Experiments with the Schwartz's reagent: General Procedure



II.53 **II.73**

To a suspension of Schwartz's reagent (459 mg, 1.42 mmol) in 11 mL of THF in a flame dried bottom round flask under nitrogen atmosphere, a solution 0.5 mmol of the disaccharide (**II.53**) after a methanolysis step, in THF (6 mL) is added. The reaction was let to stirred overnight at room temperature. The reaction was extracted with AcOEt and H_2O. The formation of the product was not confirmed by ^{1}H-NMR.

2.4.4 Experiments using Crich's methodology through N-NO formation



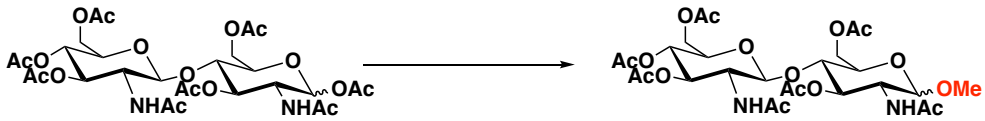
II.53 **II.78** **II.79** **II.80**

General procedure to obtain II.78 or II.81. A stirred solution of **II.53** or **II.44** in anhydrous DCM (0.1 M) was treated with anhydrous pyridine (10 equiv.) and was cooled to -10 °C. After stirring for 10 min, crushed nitrosyl tetrafluoroborate (4 equiv. per NHAc) was added in one portion to the mixture. The resultant light-yellow solution was stirred at -10 °C for 6 h then was diluted with DCM and washed with cold 1 M HCl, followed by cold saturated aqueous $NaHCO_3$. The organic layer was washed with cold brine, dried, and concentrated below 10 °C to obtain the **II.78** or **II.81** which was carried forward without any further purification.

General procedure to replace NHAc group through II.78 or II.81. A stirred solution of 18-crown-6 (173 mg, 0.66 mmol) in anhydrous DCM (1 mL) was treated with 2,2,2

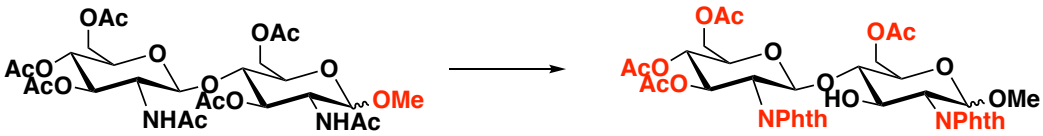
trifluoroethoxide (73 mg, 0.60 mmol) and stirred for 10 min until the solids completely dissolved and was then cooled to -10 °C. Then **II.78** or **II.81** in anhydrous DCM (0.1 M) at -10 °C was treated with the cold 2,2,2 trifluoroethoxide/18-crown-6 solution. The mixture was allowed to stir for 2 minutes for the green color to develop at which time the nucleophile (0.1 M) in DCM was added to the reaction mixture via a plastic syringe, followed immediately by addition of HBF_4 in Et_2O (0.5 equiv.). The mixture was stirred for 5 min, diluted with DCM (5 mL) and was quenched by addition of saturated aqueous $NaHCO_3$ (5 mL). The organic layer was washed with brine (5 mL), dried and concentrated to afford a yellow oil. The introduction of the nucleophile was never detected. The only product detected was the peracetylated glucose derivative.

2.4.5 Approaches using di-N-protected chitobiose derivative



II.53 **II.89**

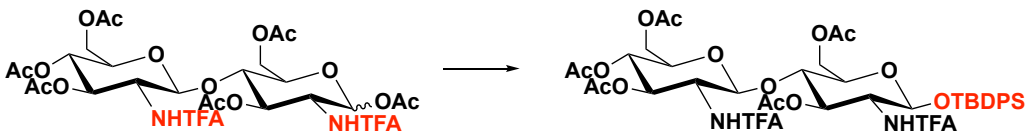
Synthesis of II.89. To a suspension of peracetylated chitobiose **II.53** (1.347 g, 1.985 mmol) in dry DCE (26 mL), was added BF_3Et_2O (0.268 mL, 2.177 mmol). After the mixture was stirred at 50 °C overnight, the resulting solution was cooled to room temperature under N_2 and dry 4 Å MS (1 g) was added. The mixture was stirred for 30 min at room temperature and then MeOH (3.0equiv, 0.3 mL) was added. After the mixture was stirred for another 2 days, it was neutralized with Et_3N, then filtered. The solid was washed with DCM-MeOH (10:1) several times. The filtrate and washings were combined, and concentrated. The residue was chromatographed on silica gel with DCM/MeOH (50: 1 v/v) as the eluent to give **II.89**, 553 mg, 43% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.93 (d, *J* = 9 Hz, 2 H), 5.27-5.22 (m, 2H), 5.04 (t, *J* = 9 Hz, 1 H), 4.56 (d, *J* = 8 Hz, 1H), 4.26-4.09 (m, 3 H), 3.89-3.82 (m, 1H), 3.70-3.67 (m, 1H), 3.46 (s, 3 H), 2.08-1.90 (m, 20H).



II.89 **II.92**

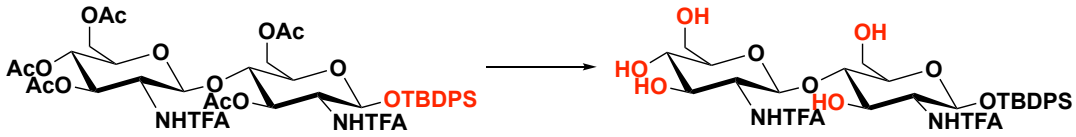
Synthesis of II.92. To a solution of **II.89** (553 mg, 0.579 mmol) in dry methanol (45 mL) was added sodium methoxide (54 mg), and the mixture was stirred overnight at room temperature, treated with Dowex 50W X-8 (H+) ion-exchange resin, and filtered. The filtrate was evaporated to dryness, the residue was dissolved in hydrazine hydrate (25 mL), and the mixture was stirred for 24 h at 120 °C, diluted with water (20 mL), evaporated to dryness, the residue dissolved in methanol (40 mL), and the solution was treated with phthalic anhydride (385 mg, 2 mmol) and triethylamine (200 mg, 2 mmol) for 3 h at room temperature. Additional aliquots of triethylamine (200 mg, 2 mmol) and phthalic anhydride (385 mg, 2 mmol) were added and stirring was continued overnight at 50 °C. The mixture was made neutral with Dowex 5OWX-8 (H+) resin, filtered, and the filtrate evaporated to dryness. The residue was treated with pyridine (25 mL) and acetic anhydride (20 mL) for 16 h at room temperature, the solution was poured into ice-cold water, and the mixture extracted with chloroform (3 x 20 mL). The extracts were combined, washed successively with HCl 1 M, saturated $NaHCO_3$ and cold water, dried, and evaporated. The residual syrup, chromatographed on silica gel using 30: 1 (v/v) toluene/ethyl acetate to give **II.92**, 162.5 mg, 34% yield. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.97-7.84 (m, 3H), 7.38-7.35 (m, 3H), 7.23-7.22 (m, 1 H), 7.16-7.14 (m, 2 H), 6.64-6.43 (m, 1H), 6.16-6.05 (m, 1H), 5.31-5.06 (m, 1H), 4.99 (s, 1H), 4.68-4.21 (m, 1H), 4.14-4.08 (m, 2H), 3.46 (s, 3H), 2.53-2.03 (m, 20H).

2.4.6 Approach towards a NAG-NAM precursors via the di-N-TFA chitobiose derivative



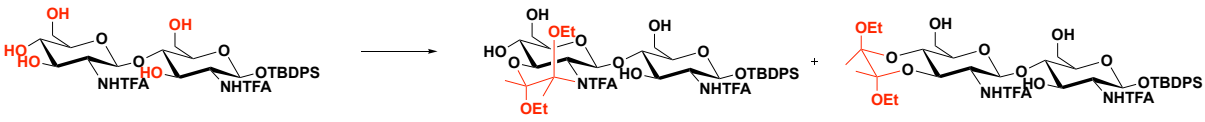
II.54 **II.93**

Synthesis of II.93. Hydrazine acetate (0.22 g, 2.45 mmol) was added to a stirred solution of **II.54** (1,75 g, 2.2 mmol) in DMF (9 mL, 0.25 M). The resulting mixture was heated for 4 h at 55 °C and then cooled to room temperature before being diluted with ethyl acetate. The organic layer was washed with brine, dried over Na_2SO_4, filtered and concentrated in vacuo to give the hemiacetal (1.6 g, quantitative) as a yellow amorphous solid. To a solution of the hemiacetal in dry DMF (8.5 mL), were added imidazole (0.44 g, 6.46 mmol) and *tert*-butyldiphenylsilyl chloride (1.4 mL, 5.4 mmol). The resulting mixture was stirred for 5 h at room temperature and then concentrated under vacuum. The residue was diluted with ethyl acetate and successively washed with water and brine. The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by flash-column chromatography on silica gel (hexane/EtOAc from 4/1 to 1/1) to give **II.93** (1.73 g, 81% yield over 2 steps) as a white amorphous solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.66 (d, *J* = 6.8 Hz, 2H), 7.58 (d, *J* = 6.7 Hz, 2H), 7.39 (m, 6H), 6.38 (bs, 2H), 5.18 – 5.10 (m, 1H), 5.04 (t, *J* = 9.5 Hz, 1H), 4.99 – 4.90 (m, 1H), 4.49 (m, 2H), 4.35 (dd, *J* = 12.5, 4.2 Hz, 1H), 4.22 (m, 2H), 4.12 (dd, *J* = 14.3, 7.3 Hz, 1H), 3.99 (m, 2H), 3.67 (m, 2H), 3.24 (d, *J* = 7.5 Hz, 1H), 2.08 (s, 3H), 2.07 (s, 3H), 2.02 (s, 3H), 1.98 (s, 3H), 1.96 (s, 3H), 1.04 (s, 9H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 170.7, 136.0, 135.7, 132.8, 132.4, 129.6, 127.7, 127.4, 100.3, 95.1, 76.2, 72.2, 71.8, 68.1, 61.7, 55.8, 26.6, 20.3.



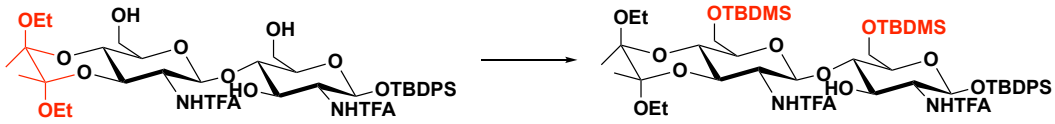
II.93 **II.94**

Synthesis of II.94. A solution of **II.93** (1.6 g, 1.63 mmol) in dry methanol (17 mL) was treated with a solution of 25 wt% NaOMe in MeOH (0.83 mL, 1.79 mmol). The reaction was stirred overnight at room temperature, neutralized with Dowex resin, filtered and concentrated under vacuum to give **II.94** (1.2 g, quantitative). ^{1}H NMR (400 MHz, MeOD) δ 7.71 (d, *J* = 6.7 Hz, 2H), 7.64 (d, *J* = 6.7 Hz, 2H), 7.39 (m, 6H), 4.63 (d, J = 8.1 Hz, 1H), 4.57 (d, *J* = 8.4 Hz, 1H), 3.93 (m, 2H), 3.75 – 3.42 (m, 7H), 3.31 (m, 2H), 2.91 (d, *J* = 8.6 Hz, 1H), 1.04 (s, 9H). ^{13}C NMR (101 MHz, MeOD) δ 159.6 (m), 137.1, 136.9, 134.4, 133.9, 131.0, 130.9, 128.6, 128.5, 117.6 (qd, J = 287.1, 11.0 Hz), 102.0, 97.1, 80.4, 78.2, 76.2, 75.1, 73.3, 71.9, 62.4, 61.2, 59.0, 57.7, 27.2, 19.9. HRMS (ESI) m/z calcd for $C_{32}H_{40}F_6N_2NaO_{11}Si$ $[M + Na]^+$: 793,2203, Found 793.2197.



II.94 II.96 II.95

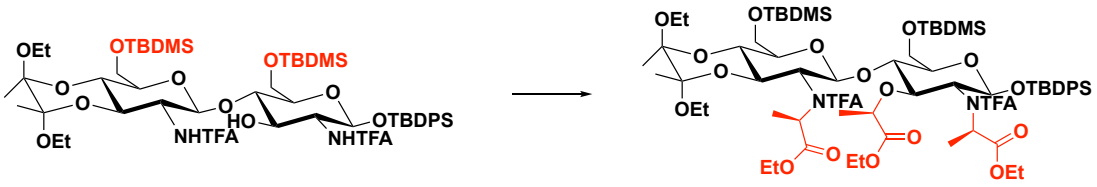
Synthesis of II.95. *p*-Toluenesulfonic acid (40 mg, 0,23 mmol) was added to **II.94** (1,2 g, 1.56 mmol) in dry ethanol (10 mL). Then, 2,3-butanodione (0.27 mL, 3.11 mmol) and dry triethyl orthoformate (1.3 mL, 7.79 mmol) were added. The mixture was heated at 60 °C for 18 h. The mixture was then basified to pH 8 by addition of triethylamine and the solvents were removed under reduced pressure. The residue was purified by silica gel column chromatography to give a mixture of **II.95** and **II.96** (1:0.3) as a white solid (1 g, 70%). ^{1}H NMR (400 MHz, MeOD) δ 7.76 – 7.67 (m, 2H), 7.67 – 7.59 (m, 2H), 7.45 – 7.28 (m, 6H), 4.62 (d, *J* = 8.1 Hz, 1H), 4.57 (d, *J* = 8.2 Hz, 1H), 4.25 (t, *J* = 10.6 Hz, 1H), 4.03 – 3.34 (m, 14H), 2.90 (d, *J* = 8.4 Hz, 1H), 1.34 (s, 3H), 1.32 (s, 3H), 1.17 (m, 6H), 1.04 (s, 9H). ^{13}C NMR (101 MHz, MeOD) δ 159.6, 159.3, 137.1, 137.0, 134.4, 133.9, 131.1, 131.0, 128.7, 128.5, 119.1, 116.2, 102.7, 102.5, 102.0, 97.1, 80.2, 77.9, 76.2, 73.3, 72.7, 70.7, 61.6, 61.1, 59.0, 57.1, 56.3, 27.3, 19.9, 19.9, 16.1, 16.0. HRMS (ESI) m/z calcd for $C_{40}H_{54}F_6N_2NaO_{13}Si$ $[M + Na]^+$: 935,3197, Found 935.3194.



II.95 II.97

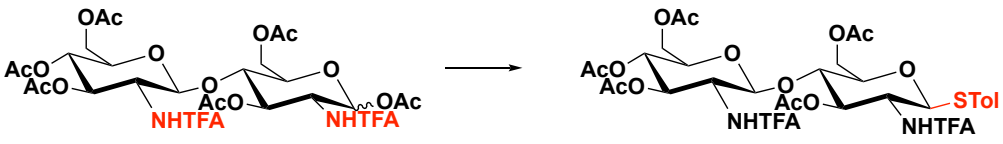
Synthesis of II.97. To the mixture of **II.95** and **II.96** (0.5 g, 0.55 mmol) in dry DCM (5 mL), were added DMAP (20 mg, 0.16 mmol), TEA (0.458 mL, 3.3 mmol) and *tert*-butyldimethylsilyl chloride (0.247 g, 1.64 mmol). The resulting mixture was stirred for 16 h at room temperature and then concentrated under vacuum. The residue was diluted with ethyl acetate and successively washed with water and brine. The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by flash-column chromatography on silica gel (from hexane to hexane/EtOAc 3/2) to afford **II.97** (0,23 g, 38%) as a white solid. ^{1}H NMR (400 MHz, MeOD) δ 7.66 (m, 4H), 7.39 (m, 6H), 4.65 – 4.55 (m, 2H), 4.33 (t, *J* = 10.6 Hz, 1H), 4.13 – 3.89 (m, 3H), 3.74 – 3.45 (m, 10H), 3.32 (m, 1H), 2.85 (m, 1H), 1.36 – 1.12 (m, 12H), 1.03 (s, 9H), 0.92 (s, 9H), 0.86 (m, 9H), 0.09 (d, *J* = 1.5 Hz, 6H), -0.06 (s,

3H), -0.13 (s, 3H). ^{13}C NMR (101 MHz, MeOD) δ (carbonyl not detected), 137.1, 137.0, 134.5, 133.7, 131.1, 130.8, 128.6, 128.5, 102.7, 102.5, 101.5, 97.0, 79.8, 77.9, 76.5, 73.3, 72.1, 70.4, 62.5, 58.9, 57.1, 56.7, 27.2, 26.5, 26.5, 19.9, 19.3, 19.1, 16.1, -4.7, -5.2, -5.2, -5.3.



II.97 II.98

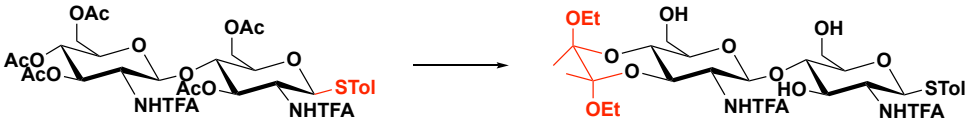
Synthesis of II.98 To a solution of **II.97** (0.12 g, 0.105 mmol) in dry DCM (1.5 mL), was added NaH (13 mg, 0.315 mmol) in an ice bath. The solution was stirred for 30 min. Then, propionic acid triflate (0.08 mL, 0.315 mmol) was added. The resulting mixture was stirred for 6 h at room temperature without evolution. The residue was diluted with ethyl acetate and successively washed with water and brine. The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by flash-column chromatography on silica gel (from hexane to hexane/EtOAc 20/1) to afford a mixture of **II.98** (0.035 g, 38%). ^{1}H NMR (400 MHz, MeOD) δ 7.65 (m, 4H), 7.38 (m, 6H), 5.39 – 5.22 (m, 2H), 4.69 (m, 1H), 4.61 (d, *J* = 7.3 Hz, 1H), 4.50 (d, *J* = 7.2 Hz, 1H), 4.18 – 3.76 (m, 11H), 3.58 (m, 6H), 3.22 (m, 2H), 2.65 (d, *J* = 9.7 Hz, 1H), 1.52 (m, 6H), 1.40 – 1.14 (m, 26H), 1.03 – 0.79 (m, 28H), 0.12 (s, 3H), 0.09 (s, 3H), -0.09 (s, 3H), -0.17 (s, 3H). ^{13}C NMR (101 MHz, MeOD) δ 175.1, 172.6, 171.5, 148.4 (*J* = 35.1, 27.2 Hz), 136.9, 136.8, 136.8, 135.1, 134.6, 130.7, 130.6, 128.5, 117.8 (*J* = 298.7, 280.5 Hz), 102.6, 102.6, 101.0, 99.1, 81.6, 78.0, 76.4, 76.0, 74.9, 74.4, 71.9, 71.0, 69.8, 65.6, 64.2, 63.0, 62.4, 62.0, 61.9, 61.2, 57.0, 56.9, 27.5, 26.8, 26.7, 20.2, 20.0, 20.0, 19.7, 19.4, 19.1, 17.7, 17.5, 16.3, 16.2, 14.6, 14.6, 14.5, -4.8, -4.9, -5.1, -5.3.



II.54 II.99

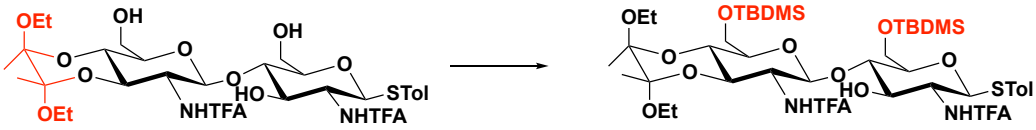
Synthesis of II.99. *p*-Tiocresol (0.09 g, 0.73 mmol) was added to a stirred solution of **II.54** (0.38 g, 0.48 mmol) in DCM (3 mL, 0.5 M). Then $BF_3.OEt_2$ (0.18 mL, 1.45 mmol) was added dropwise. The resulting mixture was stirred overnight at room temperature before quenching with $NaHCO_3$. The organic layer was washed with water and brine, dried over Na_2SO_4, filtered and

concentrated in vacuo. The residue was purified by flash-column chromatography on silica gel (hexane/EtOAc from 3/1 to 2/1) to afford **II.99** (0.24 g, 58%) as a white amorphous solid. ^{1}H NMR (400 MHz, MeOD) δ 7.35 (d, *J* = 8.1 Hz, 2H), 7.14 (d, *J* = 8.0 Hz, 2H), 5.31 (dd, *J* = 10.5, 9.4 Hz, 1H), 5.20 – 5.16 (m, 1H), 4.97 (t, *J* = 9.7 Hz, 1H), 4.79 – 4.77 (m, 2H), 4.48 – 4.42 (m, 2H), 4.10 – 4.01 (m, 2H), 3.88 – 3.74 (m, 3H), 3.70 – 3.66 (m, 1H), 2.33 (s, 3H), 2.07 (s, 3H), 2.03 (s, 3H), 2.02 (s, 3H), 1.99 (s, 3H), 1.95 (s, 3H). HRMS (ESI) m/z calcd for $C_{33}H_{38}F_6N_2O_{15}S$ [M + H]$^+$: 871.1788, Found 871.1787.



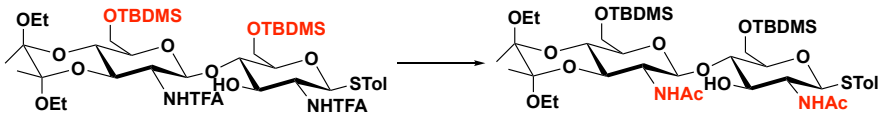
II.99 **II.100**

Synthesis of II.100. A solution of **II.99** (0.93 g, 1.28 mmol) in dry methanol (11 mL) was treated with a solution of 25 wt% NaOMe in MeOH (0.5 mL, 19.7 mmol). The reaction was stirred at room temperature overnight, neutralized with Dowex 50WX8 resin, filtered and concentrated under vacuum to afford the deacetylated product (0.65 g, quantitative). ^{1}H-NMR (400 MHz, MeOD): δ = 7.39 (d, *J* = 8.1 Hz, 2H), 7.13 (d, *J* = 8.0 Hz, 2H), 4.74 (d, *J* = 10.5 Hz, 1H), 4.62 (d, *J* = 8.4 Hz, 1H), 3.90 – 3.53 (m, 10H), 3.35 (m, 2H), 2.31 (s, 3H). *p*-Toluenesulfonic acid (28 mg, 0,16 mmol) was added to a solution of substrate (0.68 g, 1.06 mmol) in dry ethanol (6 mL). Then, 2,3-butanodione (0.19 mL, 2.13 mmol) and dry triethyl orthoformate (0.89 mL, 5.32 mmol) were added. The mixture was heated at 60 ºC for 18 h. The mixture was then basified to pH 8 by addition of triethylamine and the solvents were removed under reduced pressure. The residue was purified by silica gel column chromatography (hexane/EtOAc from 3/1 to 1/2) to give **II.100** as a white solid (0.48 g, 58%). ^{1}H NMR (400 MHz, MeOD) δ 7.38 (d, *J* = 8.1 Hz, 2H, ArH), 7.12 (d, *J* = 7.9 Hz, 2H, ArH), 4.73 (d, *J* = 10.5 Hz, 1H, H-1'), 4.61 (d, *J* = 8.2 Hz, 1H, H-1), 4.27 (t, *J* = 10.6 Hz, 1H, H-3), 3.99 (t, *J* = 10.1 Hz, 1H, H-4), 3.91 – 3.33 (m, 14H, H-2', H-6, H-2, H-4', H-3', H-6', H-5, H-5', -C*H*$_2$CH$_3$), 2.31 (s, 3H), 1.34 (s, 3H, -CH$_3$), 1.33 (s, 3H, -CH$_3$), 1.22 – 1.18 (m, 6H, -CH$_2$C*H*$_3$). ^{13}C NMR (101 MHz, MeOD) δ 159.2 (dd, J = 41.5, 37.0 Hz, COCF$_3$), 139.2 (CqAr), 133.6 (CAr), 130.7 (CAr), 117.6 (dd, J = 573.9, 287.0 Hz, COCF$_3$), 102.7 (C$_{butanodione}$), 102.5 (C$_{butanodione}$), 102.0, 101.1 (C-1), 101.0, 87.8 (C-1'), 80.5 (C-5'), 79.9 (C-4'), 77.9 (C-5), 75.0 (C-3'), 72.7 (C-3), 70.7 (C-4), 61.6 (C-6), 61.4 (C-6'), 57.1 (CH$_2$), 57.1 (CH$_2$), 56.3 (C-2'), 56.0 (C-2), 21.1 (-*C*H$_3$), 19.9 (-*C*H$_3$), 15.7 (-CH$_2$*C*H$_3$).



II.100 **II.101**

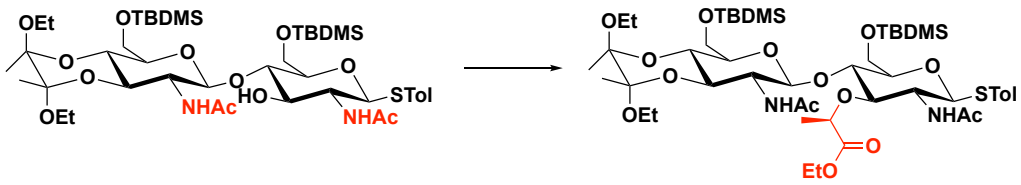
Synthesis of II.101. To a solution of **II.100** (0.48 g, 0.61 mmol) in dry DCM (4 mL), were added DMAP (23 mg, 0.18 mmol), TEA (0.52 mL, 3.69 mmol) and *tert*-butyldimethylsilyl chloride (0.278 g, 1.84 mmol). The resulting mixture was stirred for 16 h at room temperature and then concentrated under vacuum. The residue was diluted with ethyl acetate and successively washed with water and brine. The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by flash-column chromatography on silica gel (from hexane to hexane/EtOAc 4/1) to afford **II.101** (0,35 g, 56 %) as a white solid. ^{1}H NMR (400 MHz, MeOD) δ 7.36 (d, *J* = 8.1 Hz, 2H, Ar-H), 7.10 (d, *J* = 7.9 Hz, 2H, Ar-H), 4.73 (d, *J* = 10.5 Hz, 1H, H-1'), 4.63 (d, *J* = 8.2 Hz, 1H, H-1), 4.31 (t, *J* = 10.6 Hz, 1H, H-3), 4.06 – 3.92 (m, 2H, H-4, H-6), 3.86 – 3.65 (m, 8H, H-2', H-6, H-2, H-4', H-3', H-6', -CH_2CH_3), 3.57 – 3.51 (m, 3H, H-6', -CH_2CH_3), 3.37- 3.33 (m, 2H, H-5, H-5'), 2.31 (s, 3H, -CH_3), 1.36 (s, 3H, -CH_3), 1.33 (s, 3H, -CH_3), 1.19 (dd, J = 15.7, 7.1 Hz, 6H, -CH_2CH_3), 0.91 (s, 18H, Si-C(CH_3)$_3$), 0.08 (s, 12H, Si-CH_3). ^{13}C NMR (101 MHz, MeOD) δ 159.2 (dd, J = 47.0, 37.0 Hz), 139.1 (CqAr), 133.5 (CAr), 130.9 (CAr), 130.7 (CAr), 117.5 (dd, J = 286.9, 281.4 Hz), 102.7 ($C_{butanodione}$), 102.5 ($C_{butanodione}$), 101.9 (C-1), 87.9 (C-1'), 81.1 (C-4'), 80.4 (C-5'), 77.9 (C-5), 75.1 (C-3'), 72.4 (C-3), 70.5 (C-4), 63.4 (C-6), 63.0 (C-6'), 57.1 (CH_2), 57.1 (CH_2), 56.6 (C-2), 55.8 (C-2'), 26.5 (Si-C(CH3)3), 26.5 (Si-C(CH_3)$_3$), 21.1 (CH_3), 19.9 (Si-C(CH_3)$_3$), 19.3 (-CH_3), 19.2 (-CH_3), 16.1 (-CH_2CH_3), -4.7 (SiCH_3), -4.9 (SiCH_3), -5.2 (SiCH_3), -5.3 (SiCH_3). HRMS (ESI) m/z calcd for $C_{43}H_{71}F_6N_2O_{12}SSi_2$ $[M + H]^+$: 1031.3985, Found 1031.3984.



II.101 **II.102**

Synthesis of II.102. To a solution of **II.101** (0,1 g, mmol) in THF (0.5 mL) was added NaOH 2 M (2,6 mL) and the solution was heated to 80 °C for 3 h. The solution was neutralized with HCl 1 M and extracted with AcOEt. The organic layer was evaporated under reduced pressure.

The crude was dissolved with DCM (1 mL) and pyridine was added followed by Ac_2O (23 □L). The solution was stirred at room temperature overnight. The residue was purified by flash-column chromatography on silica gel (from $CHCl_3$ to $CHCl_3$/MeOH 1%) to afford **II.102** (0,07 g, 56%) as a white solid. ^{1}H NMR (400 MHz, MeOD) δ 7.36 (d, *J* = 8.0 Hz, 2H, Ar-H), 7.09 (d, *J* = 7.9 Hz, 2H, Ar-H), 4.67 (d, *J* = 10.5 Hz, 1H, H-1'), 4.47 (d, *J* = 8.2 Hz, 1H, H-1), 4.18 (t, *J* = 10.6 Hz, 1H, H-3), 4.04 (t, *J* = 10.0 Hz, 1H, H-4), 3.96 – 3.46 (m, 12H, H-2', H-6, H-6', H-2, H-4', H-3', -CH_2CH_3), 3.39 – 3.31 (m, 2H, H-5, H-5'), 2.30 (s, 3H, -CH_3), 2.01 – 1.97 (m, 6H, -CH_3), 1.35 (s, 3H, -CH_3), 1.33 (s, 3H, -CH_3), 1.18 (dd, *J* = 15.3, 7.2 Hz, 6H, -CH_3), 0.92 (s, 9H, Si-C(CH_3)$_3$), 0.91 (s, 9H, Si-C(CH_3)$_3$), 0.11 (s, 3H, Si-CH_3), 0.10 (s, 3H, Si-CH_3), 0.09 (s, 3H, Si-CH_3), 0.06 (s, 3H, Si-CH_3). ^{13}C NMR (101 MHz, MeOD) δ 173.6 (CO), 173.1 (CO), 138.6 (CqAr), 133.0 (CAr), 132.0 (CqAr), 130.6 (CAr), 103.3 ($C_{butanodione}$), 102.5 ($C_{butanodione}$), 101.8 (C-1), 88.8 (C-1'), 81.4 (C-4), 80.4 (C-5'), 77.8 (C-5), 75.7 (C-3'), 73.2 (C-3), 70.5 (C-4), 63.5 (C-6), 63.0 (C-6'), 57.1 (CH2), 57.0 (CH_2), 55.9 (C-2), 55.5 (C-2'), 26.5 (Si-C(CH_3)$_3$), 23.1 (CH_3), 23.0 (CH_3), 21.1 (CH_3), 20.0 (Si-C(CH_3)$_3$), 19.3 (-CH_3), 19.2 (-CH_3), 16.1 (-CH_2*C*H_3), -4.6 (SiCH_3), -4.8 (SiCH_3), -5.2 (SiCH_3), -5.3 (SiCH_3). HRMS (ESI) m/z calcd for $C_{33}H_{38}F_6N_2O_{15}S$ $[M + H]^+$: 905.3493, Found 905.3492.



II.102 **II.103**

Synthesis of II.103. To a solution of **II.102** (0.06 g, 0.06 mmol) in dry DCM (0.7 mL), was added NaH (4.4 mg, 0.18 mmol) in an ice bath. The solution was stirred for 30 min. Then, ethyl (S)-2-(trifluoromethylsulfonyloxy)propionate (0.046 mL, 0.18 mmol) was added. The resulting mixture was stirred for 16 h at room temperature. Since the starting material was observed by TLC, additional NaH (2.2 mg, 0.09 mmol) and ethyl (S)-2-(trifluoromethyl-sulfonyloxy)propionate (0.024 mL, 0.09 mmol) were added and stirred at room temperature overnight. The residue was diluted with ethyl acetate and successively washed with water and brine. The organic layer was dried over Na_2SO_4, filtered and evaporated under reduced pressure. The residue was purified by flash-column chromatography on silica gel ($CHCl_3$ to $CHCl_3$/MeOH 1%) to afford the **II.103** (0,02 g, 38%) as a white solid. ^{1}H NMR (400 MHz, MeOD) δ 7.35 (d, *J* = 8.0 Hz, 2H, ArH), 7.10 (d, *J* = 7.9 Hz, 2H, ArH), 4.67 (m, 2H, LacCH, H-1'), 4.61 (d,

J = 8.2 Hz, 1H, H-1), 4.20 – 4.16 (m, 4H, H-3, H-4, -CH_2CH_3Lac), 4.02 – 3.67 (m, 9H, H-2', H-6, H-6', H-2, H-3', -CH_2CH_3), 3.59 – 3.48 (m, 3H, H-4', -CH_2CH_3), 3.21 – 3.14 (m, 2H, H-5, H-5'), 2.30 (s, 3H, -CH_3), 2.01 (s, 3H, -CH_3), 1.91 (s, 3H, -CH_3), 1.43 (d, 3H, LacCH_3) 1.36 (s, 3H, -CH_3), 1.33 (s, 3H, -CH_3), 1.27 (t, 3H, -CH_3), 1.18 (m, 6H, -CH_3), 0.92 (s, 18H, Si-C(CH_3)$_3$), 0.15 (s, 3H, Si-CH_3), 0.10 (s, 3H, Si-CH_3), 0.07 (s, 3H, Si-CH_3). ^{13}C NMR (101 MHz, MeOD) δ 176.0 (COLac), 173.8 (CO), 173.7 (CO), 138.7 (CqAr), 133.4 (CAr), 131.9 (CqAr), 130.5 (CAr), 102.6 (Cbutanodione), 102.4 (Cbutanodione), 101.7 (C-1), 88.7 (C-1'), 81.6 (C-4'), 80.9 (C-5'), 77.9 (C-5), 77.0 (CHLac), 75.9 (C-3'), 73.3 (C-3), 70.2 (C-4), 62.7 (C-6), 62.6 (C-6), 61.7 (-LacCH_2CH_3), 57.0(-CH_2CH_3), 56.9 (-CH2CH3), 56.1 (C-2), 55.4 (C-2'), 26.6 (Si-C(CH_3)$_3$), 26.6 (Si-C(CH_3)$_3$), 23.3 (-$COCH_3$), 22.7 (-$COCH_3$), 21.11, 20.16, 20.1 (-CH_3), 19.6 (-CH_3), 19.4 (-CH_3), 19.2 (LacCH_3), 16.3 (-CH_2CH_3), 14.5 (LacCH_3), -4.1 (SiCH_3), -5.0 (SiCH_3), -5.1 (SiCH_3), -5.1 (SiCH_3). HRMS (ESI) m/z calcd for $C_{48}H_{84}N_2NaO_{14}SSi_2$ [M + Na]+: 1023,5079, Found 1023.5074.

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3 A chemo-enzymatic approach towards NAG-NAM containing oligosaccharides from chitosan

3.1 Introduction

Chitosan (**III.1**) and chitin (**III.2**) are structurally attractive biopolymers for chemical modifications to create the platform for new chemical entities with a broad scope of applications, ranging from material science to medicine. During the last years incredible efforts have been dedicated to the regioselective modification of these biopolymers paving the way to improved properties and tailored activities. Herein, the most recent advances on chitin/chitosan regioselective modification, reaction conditions, selectivity and the impact on its applications are highlighted. Moreover, the recent focus on chitooligosaccharides (COSs), its regioselective and chemoselective functionalization, as well as its role on biological studies, including molecular recognition with several biological targets are also covered.

3.1.1 Chitin and Chitosan

Chitin (**III.2**), the second most abundant polysaccharide after cellulose, can be obtained by extraction from crustaceans' shells and it is present in the cell wall of some fungi and algae. Chitin is constituted by of β (1-4)-*N*-acetylglucosamine (GlcNAc or NAG) and D-glucosamine (GlcN) units, Figure 3.1.[1-3]

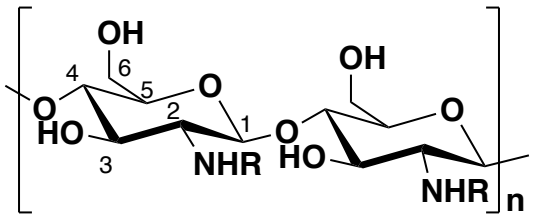


Figure 3.1 Molecular skeleton of chitosan (III.1) (R=H > 50%) and chitin (III.2) (R=acetyl).

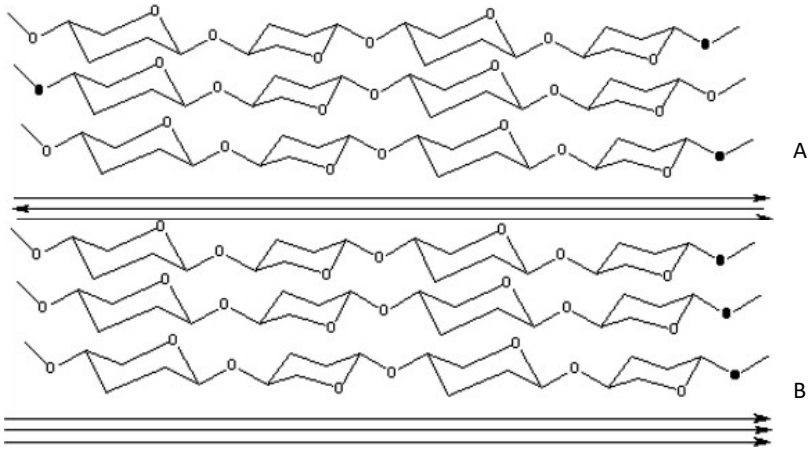


Figure 3.2 Antiparallel chain arrangement in α-chitin(A); parallel arrangement in β-chitin(B) (adapted from reference 33).

Chitosan is the copolymer, a linear heteropolysaccharide, that is obtained with the deacetylation of chitin. Chitosan, the product of the chitin's reaction[4], has frequently a DA (degree of acetylation) of less than 50%, while chitin is fully acetylated (DA=100%). Being a natural biopolymer, chitin has very interesting proprieties: it is non-toxic, biocompatible, biodegradable and it has been already explored in many distinct areas that are related with human health. It has been presented as an anti-bacterial agent[5], a fat-binding agent[6], a compound that can be used for cell protection from carcinogenesis[7], a hypocholesterolemic agent[8], an antioxidant [9], a matrix for drug release[10], a molecule that can be part of an artificial skin[11] and included in a wound dressing material[12].

Due to the poor solubility of chitin, many strategies have been developed in order to obtain derivatives, with different chemical modifications, that could have improved properties in the different application fields.[13, 14] Depending on the source and consequently the conformation of the chitin, different solubilities are associated with this polymer. Chitin has three different conformations α-, β- and ϒ-chitin that dependent on the animal source.[15] α-Chitin, which has more intramolecular forces, hydrogen-bonds associated, than β-chitin that is more frequently used due to this fact.[14] Chitin has been showed to be soluble in water but when its DA must be in a range between 45-55%.[16, 17] Several solvents have also proved to dissolve chitin and chitosan, depending on its natural source has a different solubility, as shown in Table 3.1.[18-22] In order to follow the degree of deacetylation (DA) or the depolymerization degree (DP), and accomplish the characterization of the chitoligomers many techniques have been used to provide this information, such as MALDI-TOF, ^{1}H-NMR and chromatographic techniques as GPC.[23-27] The degree of deacetylation can be calculated directly from the observation of the peak's intensity on the ^{1}H-NMR spectra as shown in equation 1.

Table 3.1List of solvents and ionic liquids used to dissolve chitin and chitosan.

Chitin (III.2)	Chitosan (III.1)
***N,N*-dimethylacetamide, 5-10%(wt) LiCl,**	Diluted acids (phosphoric, sulfuric, citric, sebacic, acetic)
***N,N*-dimethylacetamide, *N*-methyl-2-pyrrolidone, 5-8%(wt) LiCl**	Dimethylsulfoxide
Methanol saturated with calcium chloride dihydrate	*p*-Toluene sulfonic acid
1-allyl-3-methylimidazolium bromide	10-Camphrosulfonic acid
1-butyl-3-methylimidazolium acetate	1-butyl-3-methylimidazolium chloride
1-butyl-3-methylimidazolium chloride	1-butyl-3-methylimidazolium acetate
hexafluoroacetone or hexofluoro-2-propanol	1-allyl-3-methylimidazolium acetate

While the applications of chitin and chitosan in a wide range of areas, have been reviewed by several authors, the regioselective and chemoselective modification of these polymers has been scarcely reported. [3, 28-39]

$$D_{deac}\ (\%) = \left[1 - \left(\frac{\frac{1}{3}ICH_3}{\frac{1}{6}I(H2-H6)}\right)\right] \times\ 100 \qquad (1)$$

The low molecular weight chitosan has greater potential for different applications, due to its higher solubility in water. Many strategies of hydrolysis have been explored to obtain low molecular weight chitosan. This depolymerization reaction can occur by chemical hydrolysis, through the use of a strong protic acid such as nitric acid[40], diluted sulfuric acid[41] or hydrochloric acid[25, 42, 43]. Although chemical hydrolysis is the most common method to hydrolyse chitosan, other methods have been explored: 1) the enzymatic hydrolysis[44, 45]; 2) a method where the physical mechanism of depolymerization is carried out via the ultrasonic depolymerization,[24, 46, 47], which is directly affected by the geometry of the glass-wear used[24]; 3) an alternative method that use γ-irradiation depolymerization[6] and 4) a method where depolymerization is assisted by microwave[48, 49].

3.1.1.1 Selective modifications

The classic way to design novel materials from chitosan or chitin involves chemical modification reactions. For the preparation of derivatives with well-defined structures it is

crucial to manipulate chitin and chitosan in well-controlled manners. Definitely, regio- and/or chemoselective modifications with a controlled, well-defined, molecular structure is the key for the development of novel chitosan/chitin derivatives with valuable properties.[21, 22, 50, 51] In this review several examples of selective protecting group strategies on chitin and chitosan are presented, Figure 3.3.

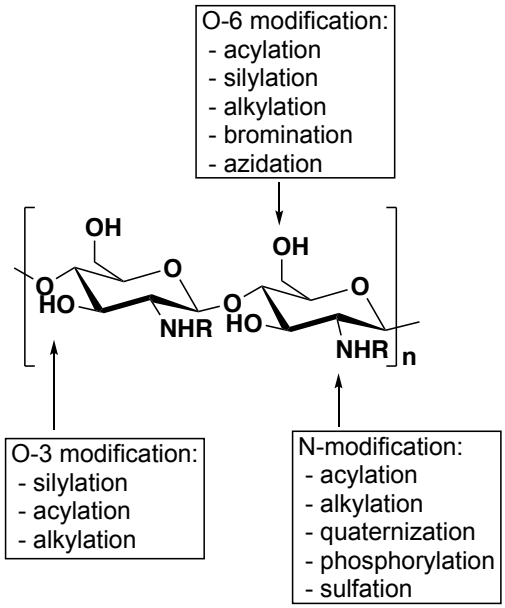


Figure 3.3 Regio- and chemoselective main modifications of chitosan (III.1).

3.1.1.1.1 Acylation

Acylated chitin and chitosan derivatives, obtained via N-, O-, or N,O-acylation, are widely used due to their versatile character and solubility properties. The acetyl group is one of the most commonly used O-protecting groups, while phthaloyl is used as N-protecting group. N-phthaloylation of chitosan is used to attain chito-derivatives with improved solubility in organic solvents while possessing a chromophore that allows easy monitoring of subsequent modifications.[52] *N*-Phthaloyl chitosan (**III.3**) can be prepared using the procedure reported by Kurita *et al*., which avoids O-6 phthaloylation.[53, 54] This procedure has also been used to introduce other groups, such as dichlorophthaloyl.[55] More recently, Ifuku *et al*. reported the N-phthaloylation of chitosan in an aqueous acetic acid solution in concentrations from 0 to 10% (v/v).[52] Despite the formation of *N*-(2-carboxy) benzoyl chitosan derivative, the intended product of *N*-Phthaloyl chitosan could be obtained with high degree of substitution (DS) after dehydration at 190 °C. The same research group has also reported the preparation of *N*-(4-

bromophthaloyl) chitosan (**III.5**), which have higher solubility in common solvents when compared with phthaloylated chitosan.[56]

The authors also reported the surface modification of chitin nanofibers, introducing maleyl (**III.8**) and naphthaloyl (**III.9**) groups, using the corresponding maleic and naphthalic anhydrides, Figure 3.4.[57] Chitosan N-carboxypropionylation (or N-succinylation) has been also reported,[58, 59] obtained using succinic anhydride under acidic conditions.[60] The formation of the N,O-disubstituted derivative, was observed in the presence of $MeSO_3H$.

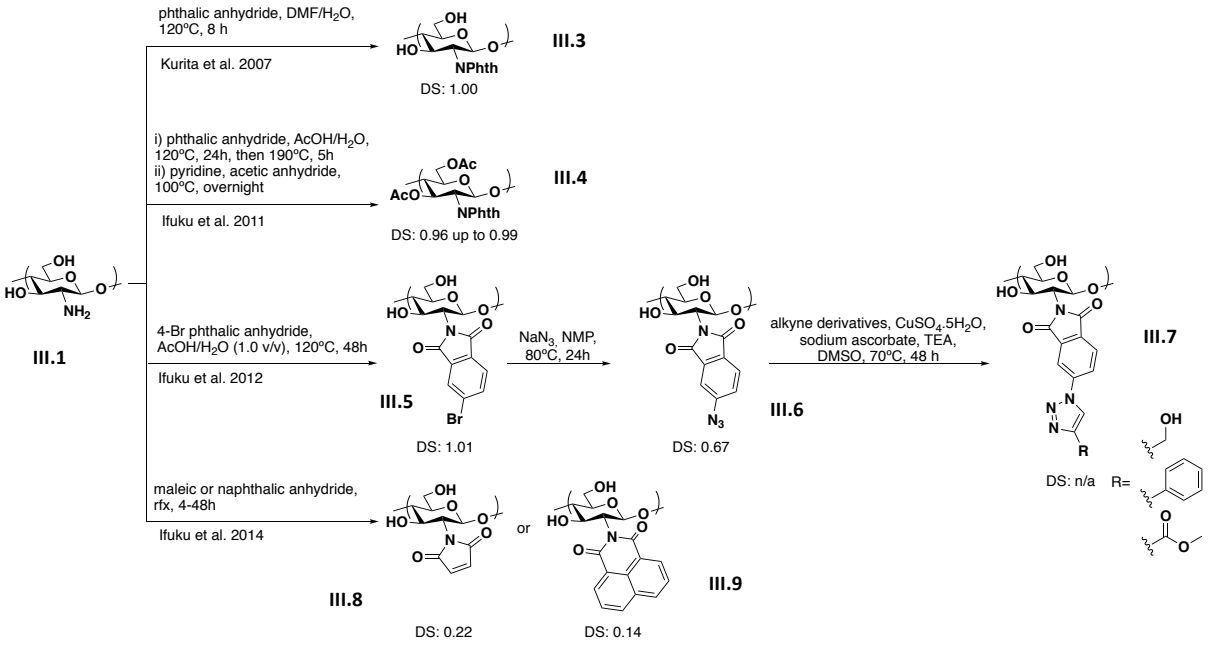


Figure 3.4 Most commonly used *N*-protecting groups.

The pivaloyl group has been used as hydroxy protecting group of primary alcohols on *N*-phthaloyl chitosan.[61] The pivaloyl group could be introduced regioselectively at O-6 using pivaloyl chloride in a homogeneous pyridine solution, yielding the product with a DS of 1.0 (**III.11**). Complete 3,6-O-dipivaloylation (**III.10**) was attained in two steps, consisting on the reaction of pivaloyl chloride in the presence of DMAP. The removal of the N-phthaloyl group could be performed in the presence of pivaloyl esters in ethanol/water, Figure 3.5.

Nishimura *et al*. could also introduce regioselectively the acetyl group at O-3 using the *N*-phthaloyl chitosan protected at O-6 with trityl group.[62] More recently, the selective preparation of *N*-(bromoacetyl)-3,6-di-*O*-*tert*-butyldimethylsilyl(TBDMS)-chitosan was reported, in order to achieve quaternized or guanidinylated chitosan derivatives.[63, 64]

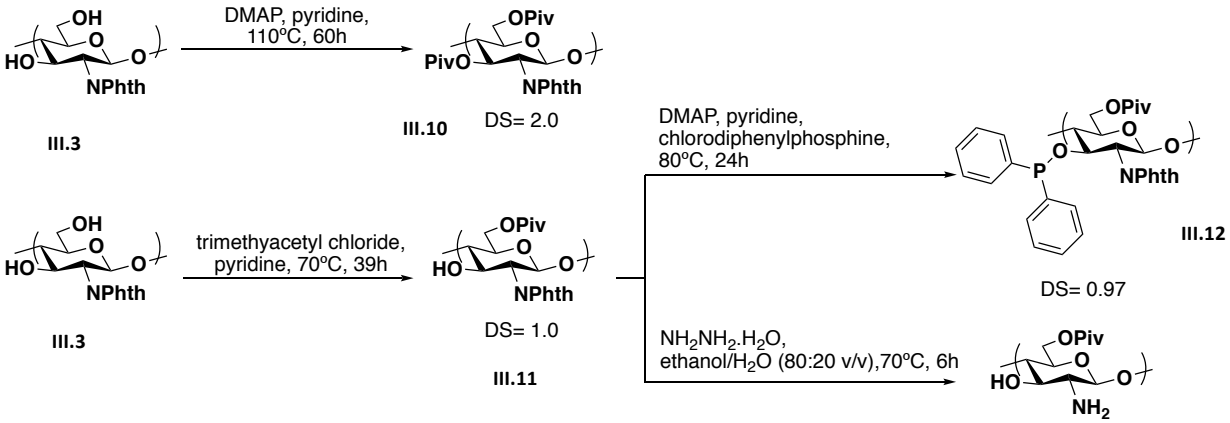


Figure 3.5 Selective pivaloylation.

3.1.1.1.2 Alkylation

Chitosan and chitin alkylation, either on amine or hydroxy groups, consists in the introduction of alkyl moieties that can contain important functionalities, such as aryl or heterocyclic structures,[65, 66] sulfonyl,[67] amine,[68] alcohol or carboxyalkyl.[69, 70] The different groups will be able to confer particular properties to the polymer.[69] Alkylation can be performed under heterogeneous or homogeneous conditions by reductive alkylation, nucleophilic substitution or Michael addition.[71] Moreover, by using the appropriate reaction conditions, it is also possible to selectively obtain the N-, O- or N,O-dialkylated products.

Carboxyalkyl chitosans are by far the most popular alkylated derivatives of chitosan. They have been employed in the development of drug delivery systems and incorporated into tissue engineering devices, due to their biocompatibility, biodegradability and osteogenesis ability.[69] Due to their amphiprotic character, O-carboxylated derivatives provide enhanced water solubility and particular chemical and biological properties. The most common N-carboxylated derivatives are the *N*-carboxymethyl-, *N*-carboxyethyl- or *N*-carboxybutyl chitosan.

In order to obtain regioselective O-6 carboxymethylation of chitosan (**III.14**), the reaction must be conducted at high alkali concentrations (at concentrations of NaOH up to 50% (m/m)), Figure 3.6A.[72] However, depending on the reaction conditions and reagent ratio, it is frequently possible to observe mixtures of N-, O- and N,O-dialkyl derivatives in the final reaction.[73] In neutral or mild basic conditions, the amine structure stands as the most reactive group, that preferentially form N-alkylated derivatives.[74] Formation of *N*- and *N*,*N*-dicarboxymethyl chitosans (**III.17**) is also dependent on the conditions used, being formed in

the presence of $NaHCO_3$ in water. Regioselective N-carboxymethylation (**III.15**) was achieved by Song *et al.* through the reaction of chitosan with chloroacetic acid in the presence of Na_2CO_3, Figure 3.6B.[75] The fully substituted *N,N*'-dicarboxymethyl chitosan (**III.17**) could be obtained by increasing the monochloroacetic acid : chitosan weight ratio.[71]

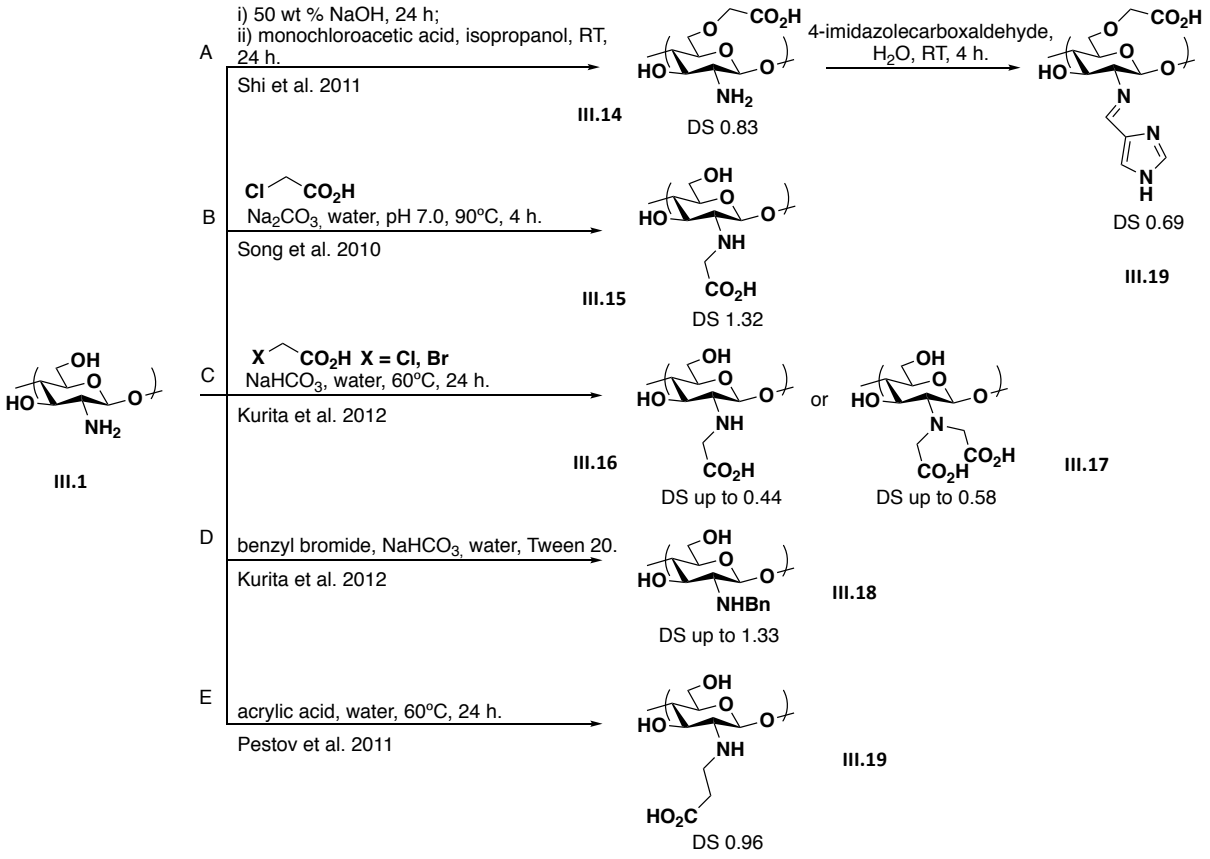


Figure 3.6Examples of O- and N-carboxyalkylation.

Kurita and Isogai studied the reaction of chitosan with different alkyl halides: monochloroacetic acid, monobromoacetic acid, 2-bromoethanol and benzyl bromide (**III.18**), in the presence of $NaHCO_3$ in water, Figure 3.6C and D. The authors refer the formation of *N*-carboxymethyl and *N*-benzyl chitosans as well as the N,N-disubstituted derivatives.[76]

Skorik *et al.* reported an approach for direct N-carboxyalkylation of chitosan in the gel state using aza-Michael addition and substitution reactions.[77] Several reagents were employed such as acrylic and crotonic acids, and α-, β-, γ-, δ- and ε-halocarboxylic acids. The authors observed that acrylic acid was an excellent and selective electrophilic reagent for N-carboxyethylation in concentrated aqueous systems.

Pestov *et al*. reported a gel-state synthesis of mono-*N*-(2-carboxyethyl)chitosan (**III.19**), where the monosubstituted derivative was attained by carboxyethylation of chitosan by acrylic acid in the presence of MgI_2 or $MgBr_2$, at a high DS and in good yields, Figure 3.6D.[78] Carboxyalkylation could also be achieved under mildly acidic medium and homogeneous conditions. *N*-(4-carboxybutyroyl) chitosan derivatives were prepared by reacting chitosan with the corresponding glutaric anhydride in a solution of 2% aqueous acetic acid–methanol (1:1, v/v).[79] The DS of this carboxymethyl chitosan could be determined by ^{1}H NMR,[74] or by potentiometric titration.[80]

Other alkyl derivatives with important biological properties were also described. Liu *et al.* described the synthesis of *O*-(3,6-hydroxyethyl) chitosan from N-phthaloylated chitosan, which after phthaloyl deprotection rendered the product.[70]

Chitin O-carboxymethylation has been also described. Huang *et al.* reported its preparation using monochloroacetic acid under basic conditions with a DS up to 1.07, although it is not clear if O-6 is predominantly attained.[81] Moreover, the authors reported that carboxymethylation of β-chitin is easier compared to reaction with α-chitin, which needs higher amounts of reactants to efficiently attain the product. Recently, the formation of *O*-carboxymethyl chitin under homogeneous conditions was described.[82] In this case, the authors were able to verify that O-6-carboxymethylated chitin was mainly produced, although O-3 substituted product was also observed.

3.1.1.1.3 Quaternization

The quaternization of chitosan is characterized by the conversion of amino group into a positively charged quaternary ammonium group or by the introduction of a quaternized moiety in the polymer backbone. This modification is usually performed to attain molecules with very particular characteristics such as improved water solubility over a broad pH range or enhanced bio-absorption properties.[83] Quaternized chitosan derivatives have been described for drug delivery or used in biomedical aplications.[83] Moreover, these derivatives show relevant biological properties, such as strong antibacterial activity.[84] Among the quaternized derivatives, *N*,*N*,*N*-trimethyl chitosan (TMC) has received particular attention. In fact, trimethylation of free amino group is by far the most preferred quaternization method, and several synthetic methods have been described, Figure 3.7.[85, 86]

Direct N-trimethylation is not a simple transformation, the traditional conditions (MeI using NMP as solvent) require the successive attack of the amine to the methylating agent in the presence of NaOH (usually at high temperature), which frequently leads to depolymerization.[85, 87, 88] Moreover, the method is not selective, yielding mixtures of *N*,*N*-dimethyl- and *N*,*N*,*N*-trimethyl-chitosans along with O-methylated products (**III.21** and **III.25**), Figure 3.7A.[89, 90]

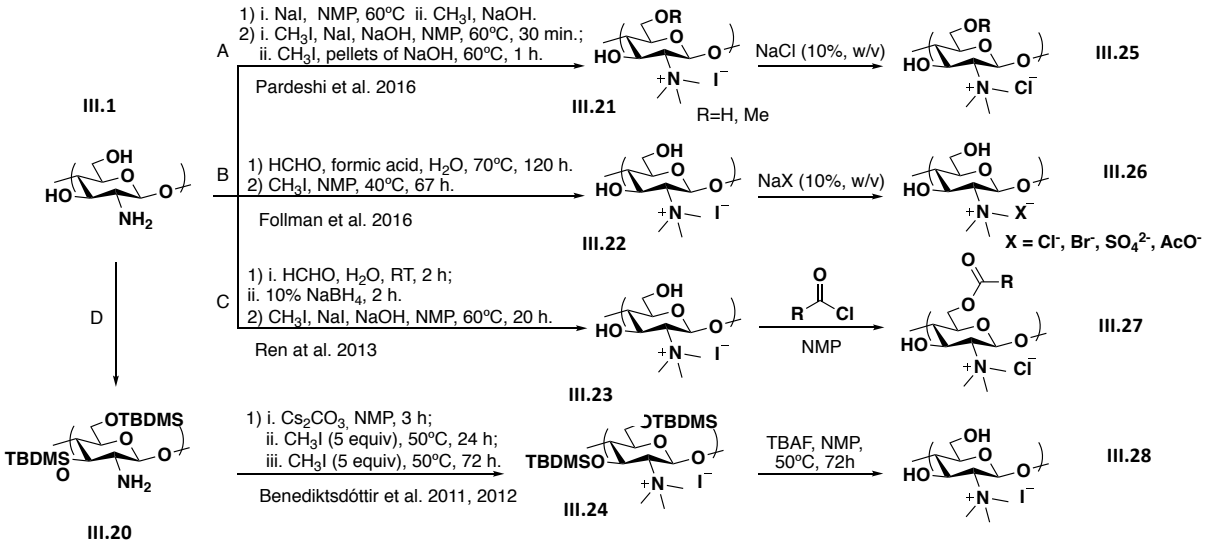


Figure 3.7 Some recently described strategies for preparation of *N*,*N*,*N*-trimethyl chitosan.

In order to overcome the described drawbacks, some groups developed parallel methodologies. The method described by Muzzarelli *et al*., which prepares TMC under basic conditions using iodomethane and *N*-dimethyl chitosan (obtained from the reaction of chitosan with formaldehyde followed by reductive conditions), is still used with slight modifications.[91] In order to prevent O-methylation, the reaction has been performed in the absence of NaOH, Figure 3.7B.[92-95] The authors confirmed by ^{1}H NMR that methylation only occurred at nitrogen atom (**III.22**).[92] In 2013, Ren *at al.* described the synthesis of N-quaternized chitosan conjugates, reacting chitosan with formaldehyde, followed by a reductive step to obtain the N-monomethyl derivative (**III.23**), Figure 3.7C.[96] The latter was then dispersed in NMP and reacted with iodomethane in the presence of NaI and NaOH, to obtain TMC.

More recently, alternative methods that guarantee the selective N-quaternization have been developed. Rúnarsson *et al.* obtained TMC with high N-quaternization degree (up to 0.92)

without concomitant O-methylation, using a DMF/H_2O solvent system, which seems to be the key for selective quaternization.[97] Other reports describe the use of O-protecting groups. Benediktsdottir *et al*. reported the synthesis of TMC and *N*-alkyl-*N*,*N*-dimethyl chitosans from the 3,6-di-*O*-TBDMS chitosan (**III.20**), allowing full substitution of the free amino groups, Figure 3.7 D.[63, 64] It was stated that 3,6-di-O-TBDMS intermediate enhanced the solubility of chitosan in organic solvents, providing homogenous reaction conditions. Thus, full N,N,N-trimethylation of chitosan (**III.24**) was attained using methyl iodide in the presence of mild base such as Cs_2CO_3. After desilylation, the authors verified the inexistence of N,N-dialkylation, N-monoalkylation or O-alkylation products. Moreover, *N*-propyl-, *N*-butyl- and *N*-hexyl-*N*,*N*-dimethyl chitosan derivatives were prepared by reductive alkylation using the corresponding aldehydes, followed by dimethylation using dimethyl sulfate in dichloromethane.[98]

More recently, in order to study the activity against Gram positive and Gram negative bacteria, the same group described the preparation of several *N*-alkyl-*N*,*N*-dimethyl chitosans, as well as the *N*,*N*-dialkyl-*N*-methyl chitosan derivatives, using the same methodology.[84] However, for *N*,*N*-dialkyl derivatives the conditions used for quaternization of TMC were not successful. The authors proposed that N,N-dialkylated-3,6-O-di-TBDMS derivatives possess a bulky structure, hindering the subsequent methylation. After optimization, the quaternization was achieved using MeI in the presence of NaOH (in NMP). The reaction was repeated twice in order to obtain a higher degree of quaternization.[84]

The group also employed a resourceful strategy to attain structures where the quaternary groups were distanced from the chitosan backbone by alkylic spacers having different lengths, Figure 3.8.[63] The compounds were designed in order to determine which structural features have impact over antibacterial activity. The group studied how the degree of acetylation, molecular weight, quaternary group or the positioning of the cationic charge, affects the activity.[63] It was observed that TMC showed the highest antibacterial activity followed by the derivatives with trimethylammonium as the quaternary group. However, increasing the spacer length, led to a decrease of biological activity, Figure 3.8.

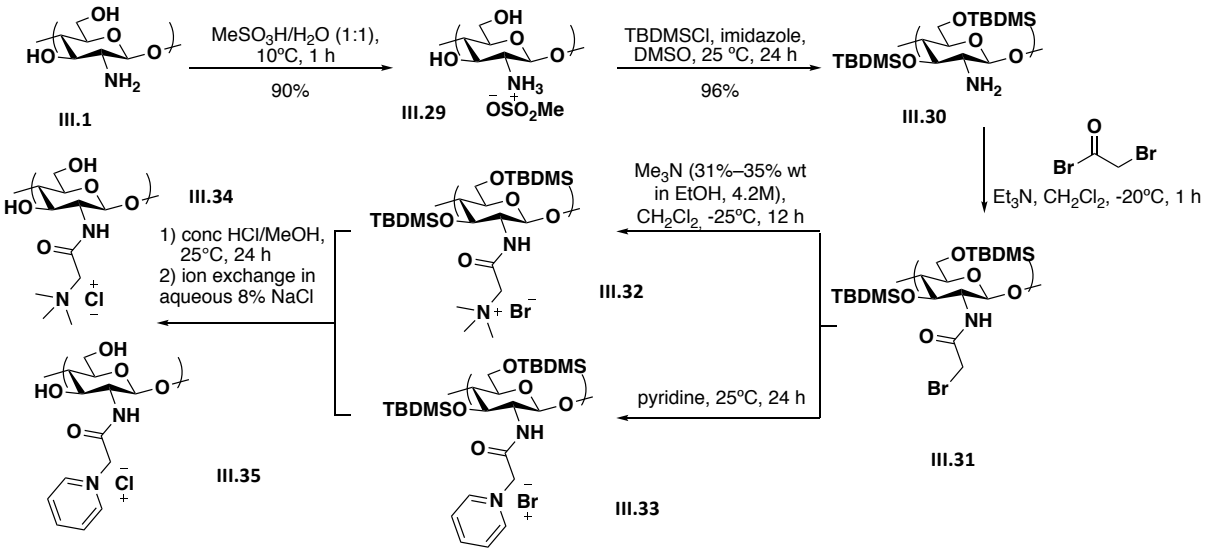


Figure 3.8 Quaternized derivatives described by Sahariah *et al.*

It has also been described that the bactericidal activity of quaternized chitosan derivatives is influenced by the counterion. Normally, under the usual quaternization conditions, the counterion of TMC is the iodide anion. Follmann *et al.* compared the activity of *N*,*N*,*N*-trimethyl chitosan acetate, sulfate, chloride, bromide and iodide against *E. coli* and *S. aureus*.[95] *N*,*N*,*N*-trimethyl chitosan sulfate and *N*,*N*,*N*-trimethyl chitosan acetate displayed the highest activities, while among the derivatives with halide counterions, *N*,*N*,*N*-trimethyl chitosan chloride presented the best results. The authors proposed that bactericidal activity is influenced by the distinct shielding degrees of the electrostatic interactions induced by the different counterions.[95] Recently, a "green" approach described the selective N,N,N-trimethylation performing the reaction in a deep eutectic solvent (DES)/urea system.[99] Other N,N,N-trimethylated derivatives can be prepared, such as the chitosan-*N*-2-hydroxypropyl trimethyl ammonium chloride, which was prepared by reaction of chitosan and glycidyl trimethylammonium chloride in isopropyl alcohol.[100]

Quaternization of chitin can be attained by hydroxy group modification. Chitin can be dissolved in a NaOH/urea aqueous solution, and quaternization could be achieved under homogeneous reaction adding 2,3-epoxypropyl-trimethylammonium chloride to the chitin solution.[101] However, quaternized chitins were obtained with moderate degrees of substitution (from 0.25 to 0.42) by changing the molar ratio of epoxide.

3.1.1.1.4 Sulfation

Sulfated chitin and chitosan derivatives exhibit important biological properties such as antithrombotic and antibacterial activities. Moreover, they have been applied in different areas, such as metal ion adsorption or drug delivery systems.[102] Thus, the chemo- and regioselective control of sulfation is determinant to access compounds with specific characteristics.[103] Unfortunately, and despite the wide number of articles covering chitin/chitosan sulfation, there is still a lack of reports with a comprehensive study on selective sulfation. Sulfated chitosans have been prepared using different reagents including chlorosulfonic acid ($ClSO_3H$), sulfuryl chloride, sulfuric acid, SO_3 or sulfamic acid.[102-104] However, the sulfation conditions affect the position and degree of substitution and several sulfation patterns are usually obtained.[102, 103, 105] In order to attain sulphated derivatives in high selectivity, improved methodologies have been developed, including the optimization of the standard conditions or the sulfation of N- or O-protected chitosans, Figure 3.9.[105, 106]

6-O-Sulfated chitosans (**III.36**) are relevant biologic targets, and its preparation in high selectivity is of great importance.[103, 107, 108] 6-*O* Sulfated chitosan (**III.36**) has been obtained using a chlorosulfonic acid under acidic conditions.[107] These conditions guarantee the protonation of amine group, leaving the most reactive hydroxy at O-6 free to react. In 2012, Wang *et al.* reported the chemo- and regioselective sulfation of chitosan to attain the 6-O, 3,6-O and 2-N,6-O-sulfated derivatives (**III.39**), Figure 3.9A.[109] The 6-O- and 3,6-O-disulfated (**III.37**) derivatives were obtained by tuning the reaction conditions using chlorosulfonic acid. More recently, Ding *et al*. reinforced that selective 6-O-sulfated (**III.36**) chitosan could be attained using chlorosulfonic acid/sulphuric acid system, Figure 3.9B.[103] Moreover, reaction of chitosan formate with chlorosulfonic acid yielded predominantly the 6-O-sulfated chitosan, either under homogeneous or heterogeneous conditions.[105]

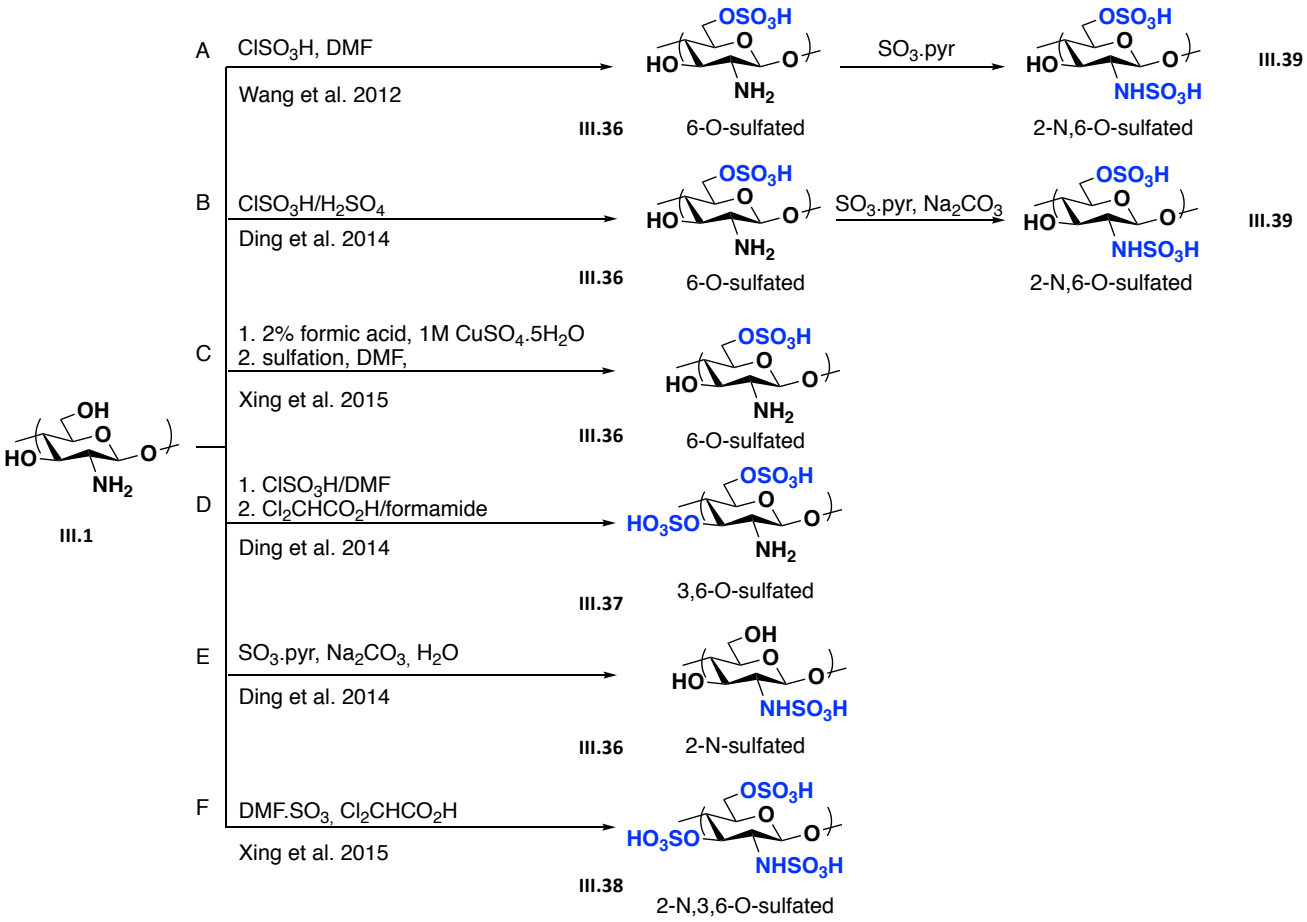


Figure 3.9 Selective sulfation strategies.

Other procedures were also described, such as the one from Xing *et al*. on the exclusive production of 6-O-sulfated chitosan (**III.36**) in a two-step reaction, Figure 3.9C.[107] Firstly, a solution of chitosan in 2% formic acid was reacted in the presence of 1 M $CuSO_4 \cdot 5H_2O$, followed by reaction with SO_3·DMF. It seems that under these conditions, the copper salt prevents reaction at O-3. It is also commonly assumed that 3,6-O-disulfated chitosan (**III.37**) derivative can be obtained using chlorosulfonic acid in the presence of a dichloroacetic acid (DCAA)/formamide system, Figure 3.9D.[103, 110] Moreover, in order to assure selective O-sulfation, some authors used phthaloylated chitosan with DMF·SO_3, followed by deprotection with hydrazine, Figure 3.10A. Otherwise, O-3-sulfated derivative was obtained from the 3,6-di-*O*-sulfochitosan which was heated in a mixture of NMP/water. In fact, according to previous reports, the methods that ensure selective modification are those that employ suitably protected starting materials. Nishimura *et al.* reported the synthesis of 2-N,3-O-sulfated chitosan (**III.42**) and O-3-sulfated chitin from the *O*-6 trityl derivative (**III.41**), Figure

3.10B. The authors were able to obtain a degree of sulfation of 1.7 for 2-*N*,3-*O*-sulfochitosan (**III.42**).[106]

Chemoselective N-sulfation can be performed using the SO_3.pyridine complex in the presence of mild base such as Na_2CO_3, Figure 3.9E, whereas 2-N,6-O-sulfated chitosan (**III.39**) is obtained from O-6 sulfated derivative involving the SO_3.pyridine complex, Figure 3.9A and B.[103, 111]

Fully sulfated chitosan can be prepared by a combination of the already described procedures. Xing *et al.* prepared the 2-N,3,6-O-disulfated derivative (**III.38**) using the DMF·SO_3 complex in DMF/DCAA or DMF/formic acid mixtures, Figure 3.9F.[107] Chitin regioselective sulfation has been also described.[112] For chitin the concern resides on the discrimination of both hydroxy groups, although the methodologies used for chitosan can be applied in this case.[106] The sulfonyl group can also be distanced from the chitosan backbone by an alkylic spacer, which differentiate polymer characteristics.[113] Thus, *N*-(2-sulfoethyl)chitosan could be selectively prepared using 2-bromoethanesulfonate, while Jiang *et al.* described the preparation of the *N*-(2 sulfopropyl) chitosan by Michael addition under mild conditions.[67]

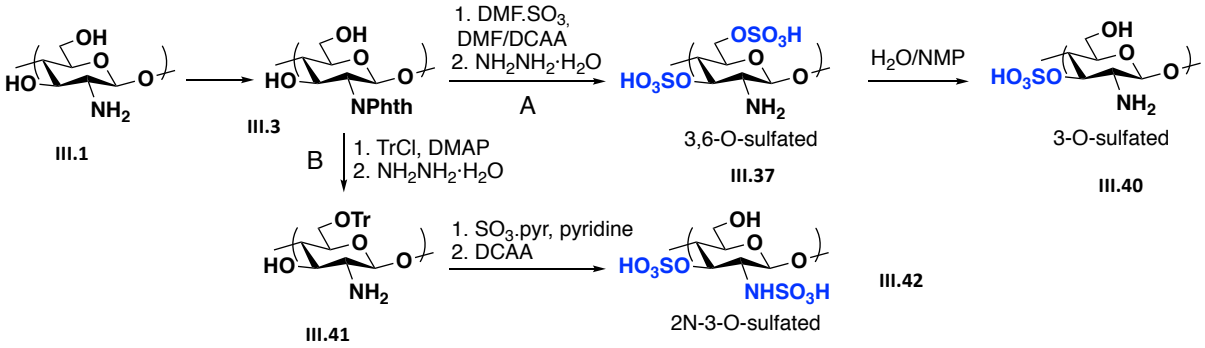


Figure 3.10 Sulfation methods employing O- or N-protected derivatives.

3.1.1.1.5 2-Azidation

2-Azido chitosan is a functional precursor that can be used in click chemistry transformations.[114, 115] The transformation of the amine group into the azide moiety can be performed using sodium azide or by diazo transfer using trifluoromethanesulfonyl (triflyl or Tf) azide or imidazole-1-sulfonyl azide hydrochloride in the presence of $CuSO_4.5H_2O$.[114, 115] Zhang *et al*. described the synthesis of the 2-azide derivative from 6-*O*-trityl chitosan (**III.41**), due to the poor solubility of chitosan in organic solvents, Figure 3.11A.[114] The desired azide moiety was introduced by $CuSO_4$-catalyzed diazo transfer in high yield, although the Cu salts

could not be completely removed. After detritylation, the desired 2-azide chitosan (**III.44**) was obtained in high yield. Marzaioli *et al*. described the synthesis of azidated oligomers. In this case, the reaction was conducted in phosphate buffer 0.5 M at pH = 7 with imidazole-1-sulfonyl azide hydrochloride, which is a safer alternative to triflyl azide, Figure 3.11B.[116] Kulbokaite *et al.*, described the azidation of chitosan using different methods, Figure 3.11C.[115] The authors reported that the introduction of sodium nitrite led to depolymerization of chitosan, even using low amounts and under mild reaction conditions. Moreover, the authors concluded that the use of imidazole-1-sulfonyl azide hydrochloride was the most effective reagent when compared to triflyl azide.

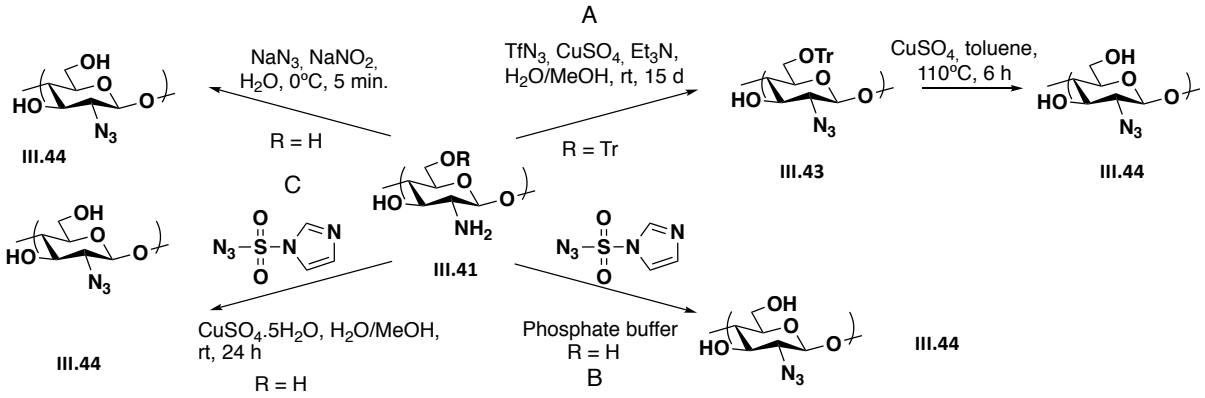


Figure 3.11 Protocols developed to convert the amino group into azide.

3.1.1.1.6 Phosphoryl and phosphonation

In the last years, phosphorylated biopolymers have received great attention due to their vast range of properties and applications, including the corresponding chitin and chitosan derivatives that possess particular chemical and biological relevance.[117] Several studies have been reported on chemo- and regioselective phosphorylation of chitin and chitosan, in order to attain well-defined and highly functionalized structures.[118] It is commonly accepted that fully phosphorylated compounds can be obtained using orthophosphoric acid and urea in DMF at high temperatures, although a recent study suggests that under these conditions only *N*-phosphoryl chitosan (**III.45**) is obtained, Figure 3.12A.[118, 119] According to the authors *N*-phosphoryl chitosan can also be prepared using the $H_3PO_4/(EtO)_3PO/P_2O_5$ system, Figure 3.12B.[119]

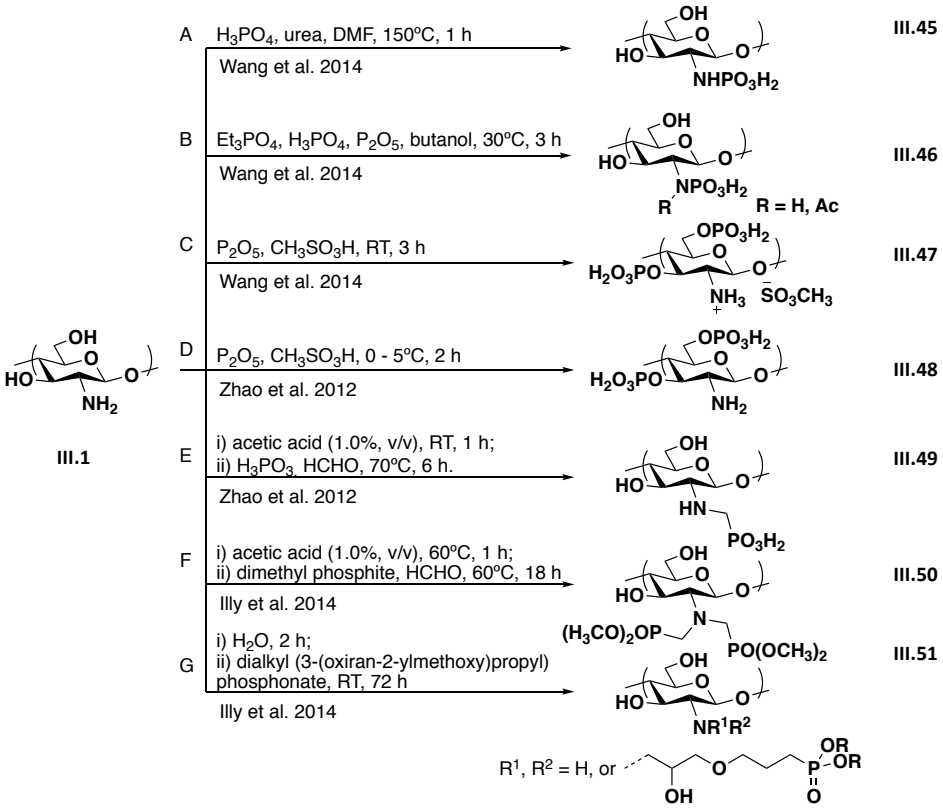


Figure 3.12 Examples of phosphoryl and phosphonation methods.

The authors also used phosphorous pentoxide in methanesulfonic acid conditions, to promote phosphorylation at O-6 and O-3 (**III.47**), due to the lack of reactivity of protonated amine, Figure 3.12C.[117] In fact, O-phosphorylated derivatives are commonly formed under these conditions, where methanesulfonic acid assists chitosan dissolution and acts as catalyst for the esterification reaction, Figure 3.12D.[120] Other methodologies were used towards phosphorylated compounds such as *N*,*O*-diethoxy phosphoryl chitosan, which was obtained *via* an Atherton-Todd reaction.[121, 122] A similar approach was employed to prepare chitosan-O-isopropyl-5'-O-d4T monophosphate conjugate.[122] However, in this case the reaction was performed selectively at the amine, since the phosphoramide moiety was assembled from 6-*O*-trityl chitosan, to yield the 6-*O*-trityl chitosan conjugate, which after removal of the trityl group, conducted to the desired product.[122]

Phosphonate and phosphonic acid groups are also commonly introduced in chitosan and chitin backbone.[120, 123] These derivatives are less sensitive towards hydrolysis than phosphates, having a wide range of applications.[123] *N*-Methylene phosphonic acid chitosan (**III.49**) is typically formed by reacting the polymer with formaldehyde and phosphorous acid usually above 70 ºC, Figure 3.12E, despite the possible formation of N,N-dimethylene

derivative.[124] *N*-Methylene phosphonic chitosan could also be prepared under microwave conditions.[125] Other protocols were already described for phosphonation of hydroxy and amine groups.[123, 126] Illy *et al.* reported the synthesis of aminoalkyl phosphonic acid chitooligosaccharides using different approaches, Figure 3.12F and G.[120] The authors also attempted the hydrolysis of the phosphonate diester to free phosphonic acid for both products, although it was only possible in the case of phosphonated derivatives prepared via Kabachnik–Fields reaction.

3.1.1.1.7 Other regioselective O-modifications

Taking advantage of the different inherent reactivity of chitin and chitosan hydroxy groups, e.g. higher reactivity and less sterical hindrance of 6-OH than 3-OH, several methods have been developed to regioselectively modify the polymer backbone. The most commonly used hydroxy protecting groups are acyl,[62] *p*-toluenesulfonyl (tosyl),[127] triphenylmethyl (trityl),[128-130] or silyl groups, such as trimethylsilyl (TMS) or TBDMS, Figure 3.13.[131, 132] The primary alcohol at O-6 is very versatile, and its adequate protection can increase polymer organosolubility or enable particular transformations. It can be regioselectively protected and transformed into different functionalities, such as halide,[133] azide,[56, 134, 135] carboxylic acid,[136] amine,[137] thiol,[138, 139] or to diethylphosphite.[127]

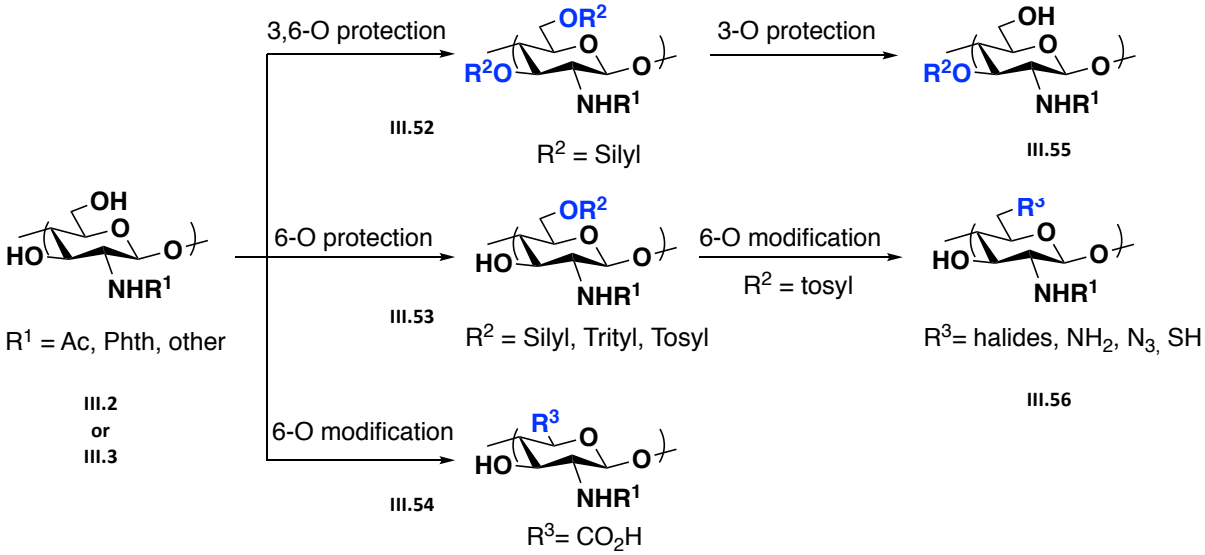


Figure 3.13 General scheme for O-modification.

A common strategy consists in the regioselective protection of O-6 position with bulky groups such as trityl group or silyl moieties like TBDMS or *tert*-butyldiphenylsilyl (TBDPS), leaving the

position O-3 free for further protection. Some of these procedures employ N-phthaloylated chitosan, which possesses increased solubility in organic solvents. In fact, O-6 protection allows subsequent synthetic modifications at amino group. Silyl groups can be introduced under mild conditions at O-6, and are stable under several reaction conditions (although they can be sensitive to acidic environments). Binette *et al*. described the regioselective protection of *N*-phthaloyl chitosan with TBDMS and TBDPS, using imidazole(Im)/DMF or dimethylaminopyridine (DMAP)/pyridine systems.[140] After careful optimization, it was verified that the best conditions for TBDMS introduction at O-6 consists of Im/DMF (with a DS of 0.92), while TBDPS needed a higher number of equivalents to attain a DS of 0.86, possibly due to its bulkiness. The chemoselective synthesis of 3,6-di-*O*-TBDMS chitosan without using common N-protecting groups, was performed using the mesylate salt of chitosan and TBDMSCl/Im in DMSO (DS of 1.94). It was verified that chitooligomers can only be fully silylated by sequential treatment with TBDMSCl and TBDMSOTf, in order to silylate the sterically hindered secondary alcohol.[51, 141] The 3,6-O-silyl protection enhances the solubility of chitosan in organic solvents, allowing subsequent chemical modifications. Sulfated chitosans were easily prepared under homogenous conditions using 3,6-*O*-TMS chitosan that significantly enhanced the reactivity of chitosan in organic solvent.[142] Watanabe *et al.* introduced triethylsilyl (TES) and triphenylsilyl (TPS) groups on β-chitin.[132] 3,6-Di-O-triethylsilylation was attained in DMAP/pyridine (DS of 1.98), while 3,6-di-*O*-triphenylsilyl derivative was attained in Im/pyridine (DS of 1.73).

Like silyl groups, trityl increases the polymer solubility in organic solvents allowing subsequent modifications.[128-130] It is usually introduced at O-6 using trityl chloride in pyridine, and is easily cleaved under mild acidic conditions.[130] In 2010, a controlled regiospecific structural modification of chitin and chitosan was reported by Kurita *et al*.[128] The three reactive positions were differentiated in order to establish a protecting and deprotecting sequence with high selectivity. In chitin, the benzyl, trityl and acetyl moieties were evaluated as protecting groups for O-3, O-6 and N-2 positions, respectively. Thus, chitin (**III.2**) was firstly silylated to form 3,6-di-*O*-TMS derivative (**III.57**) that was further reacted with trityl chloride to afford 6-*O*-trityl-chitin (**III.58**). This derivative was benzylated to give 3-*O*-benzyl-6-*O*-trityl-chitin (**III.59**), in which the benzyl, trityl, or acetyl groups could be selectively removed to afford three different derivatives with a free hydroxy or amine group, Figure 3.14. Using a similar methodology, 3-*O*-benzyl-6-*O*-trityl-*N*-phtaloyl chitosan was prepared. The resulting

fully protected products were selectively deprotected to afford the corresponding products.[128] Interestingly, it was observed that the chitin type (α or β) influences some chemical modifications.[129]

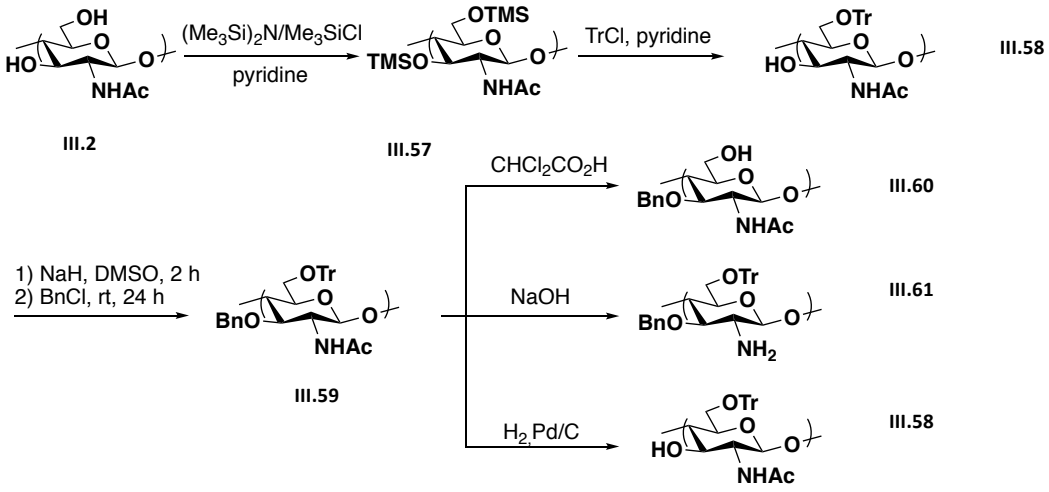


Figure 3.14 Protection of O-6 with trityl group.

Umemura *et al.* verified that the functionalization of hydroxy groups of squid β-chitin with trityl or benzyl groups proceeded easily than those of shrimp α-chitin.[129] While for α-chitin the 6-*O*-trityl derivative was prepared from the trimethylsilylated chitin, the β-chitin exhibited higher reactivity and direct O-6 tritylation was achieved in a quantitative way. 3,6-O-Dibenzylation of β-chitin was also accomplished in a simple one-step reaction.[129]

Recently, Sugita *et al.* reported the introduction of peracetylated D-glucosamine branches into chitin backbone.[143] The glycosylation proceeded with the glucosamine oxazoline as donor and the trimethylsilyl chitin derivative as acceptor.

Tosyl group is a very versatile protecting group for O-6 position due to its good leaving character. Thus, O-6 tosylated derivatives are useful intermediates for subsequent reactions. The preparation of tosyl derivatives comprises the reaction of chitosan or chitin with tosyl chloride in pyridine or in a DMAc (N,N-dimethylacetamide)/LiCl solution, respectively.[127, 134, 139] The recently described methods for chitin tosylation are an alternative to that described by Kurita *et al.*, where deacetylation was observed.[144] Several nucleophiles such as sodium iodide,[144] sodium azide,[56, 134, 135] or potassium thioacetate[138] were used to replace the tosyl group in order to produce iodo-, azido-, or mercapto-chitin and chitosan derivatives, respectively. In fact this methodology can be applied to produce relevant materials, such as a chitosan derivative possessing a cyclodextrin at O-6, which was obtained via nucleophilic

substitution of the tosyl group by an ethylenediamine substituted cyclodextrin. Removal of acetyl groups on the chitin main chain yielded the C-6 substituted cyclodextrin chitosan.[145] Other versatile derivatives could also be obtained from O-6 tosylated chitin, such as 6-*O*-ethyl benzoate-, 6-deoxy-diethyl malonate- and 6-(deoxydiethyl) phosphate chitin.[56, 134, 135]

The O-6 modification is also widely used to create amphiprotic chitosan derivatives, which are compounds that are water soluble and can be used in different several applications.[69, 70] Usually, the amphiprotic character can be achieved by carboxymethylation of O-6 (already described) or by transforming this position into a carboxylic acid.[67] Conversion of the O-6 primary hydroxy into a carboxylate group is usually achieved under oxidative conditions using TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl radical) and NaClO in the presence of NaBr, described for both chitin and chitosan.[136, 146] For chitosan, poor yields and an oxidation degree of 40% were obtained, although carboxylate groups could be identified by FTIR and NMR, along with significant depolymerization that took place during the oxidation procedure. The O-6 oxidation was also attempted using a laccase-TEMPO system.[147]

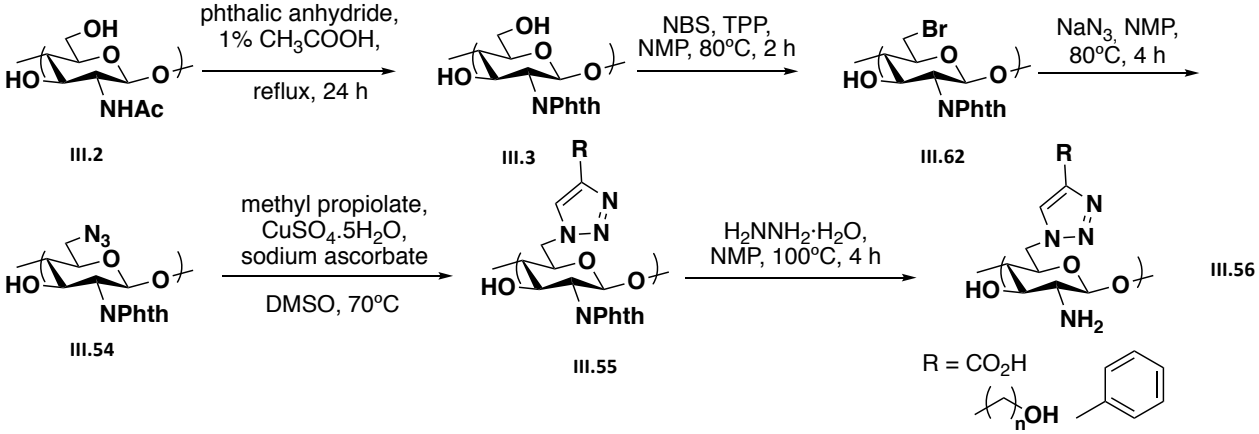


Figure 3.15 Example a versatile O-6 modification.

The carboxylic acid moiety can be substituted by a bioisostere group, such as triazole, which can be constructed by click chemistry.[56, 135] Ifuku *et al.* described the O-6 modification of the *N*-phthaloyl chitosan, which was brominated using *N*-bromosuccinimide and triphenylphosphine followed by azidation with NaN_3, Figure 3.15. The azide moiety at the C-6 position (**III.54**) was then successfully converted to a 1,4-triazole linker (**III.55**) with an appropriate R group, by a Huisgen cycloaddition between 6-azido-6-deoxy-*N*-phthaloyl-chitosan (**III.56**) and an adequate propiolate in the presence of Cu(I) catalyst.[135] The azide

moiety placed at O-6 (**III.54**) can also be transformed in an amine group (**III.58**) via formation of a triphenylphosphinimine intermediate that is hydrolyzed using aqueous hydrazine, with simultaneous removal of the N-phthaloyl group, Figure 3.16. This sequence gave 6-amino-6-deoxy-chitosan, which, unlike chitosan, is soluble in water at neutral pH.[133] Another method to produce 6-amino-6-deoxy chitosans also involves the formation of the azide intermediate obtained from the 6-*O*-tosyl-*N*-phthaloyl chitosan. However, in this case, the authors performed a preliminary hydrazinolysis step followed by reduction of azide using $NaBH_4$ in DMSO.[137]

Recently our group reviewed all the methods for selective modification of chitin and chitosan recently reported.[148]

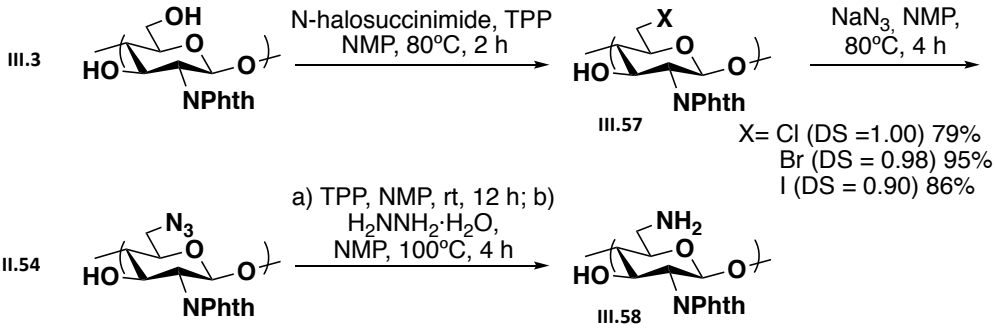


Figure 3.16 Synthesis of 6-amino-6-deoxy-chitosan.

The unique structure of chitin and chitosan, when combined with a properly controlled chemical and/or enzymatic transformation, improves the design of novel materials with refined macromolecular structures and specific properties.[148] The classic way to design novel materials from chitin or chitosan involves its chemical modification, and the preparation of chitin or chitosan derivatives with well-defined structures is crucial to manipulate chitin and chitosan in well-controlled manners. Undoubtedly, their chemo- and/or regioselective modification allied to a controlled molecular structure is the key for the progress on novel chitin/chitosan derivatives possessing valuable properties. [21, 22, 149, 150]

The use of high molecular weight biopolymers to attain smaller molecules, as an alternative to classical glycosylation methods as emerged as a new breadth to attain glycostructures via a reversed synthetic approach. Indeed, COSs obtained from chitin or chitosan, are highly attractive molecules for further chemical modification.[148] (see Chapter 2)

However, COSs have been scarcely explored as potential building blocks to attain advanced and tailored glycostructures with potential application in glycobiology. [151-153]

The structural resemblance between chitin and the carbohydrate skeleton of peptidoglycan (PGN), Figure 3.17, - the major component of the bacterial cell wall, led us to recently explore chitobiose, obtained from chitosan, as starting material for PGN synthesis.[153]

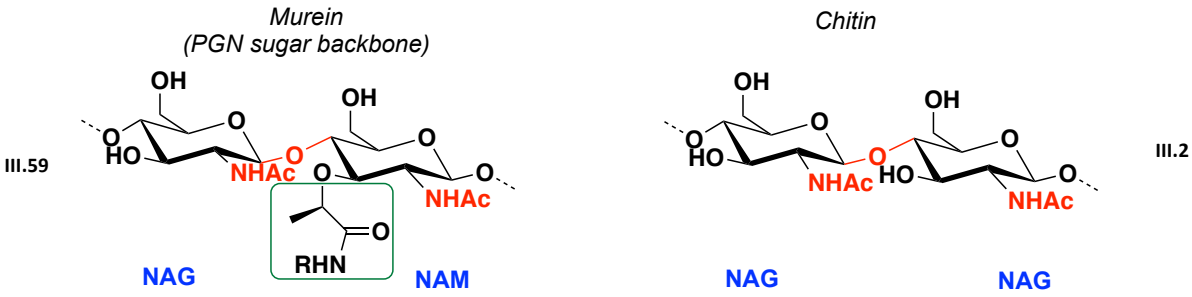


Figure 3.17 Comparison between Murein and Chitin structures.

3.2 Results and discussion

3.2.1 Goals

Since chitosan and PGN share the same basic carbohydrate backbone, and on the follow-up of our recent work on chitobiose regioselective modification, we anticipated that chitosan of high polymerization degree would be an attractive starting material for the preparation of NAG-NAM containing oligosaccharides, that can be further manipulated to construct PGN mimetics useful as probes in biological studies.[154-157]

It was envisaged that a properly functionalized, chemically modified, chitosan would be an attractive starting material for attachment of the Lac unit in the chitosan backbone. Via a synthetic strategy and after sequential removal of the protecting groups, chitosan would provide a carbohydrate similar to PGN as 1) it would be recognized as PGN by different PGN interacting proteins, such as the Drosophila PGRP-SA receptor; 2) it could be used in the preparation of smaller oligosaccharides mimetics of NAG-NAM units obtained after recognition of the modified polymer by hydrolytic enzymes such as lysozyme or mutanolysin.[158-160] These enzymes can hydrolyze efficiently the β-(1,4) glycosidic bond in the natural PGN substrate, between NAM and NAG residues[161-165] and, in certain conditions and to some extent, chitin, Figure 3.18.[166] Furthermore, the combination of chemical and enzymatic approaches have become an extremely attractive option, relatively to the traditional orthogonal synthesis, in the synthesis of complex and biological important molecules.[161, 167-170]

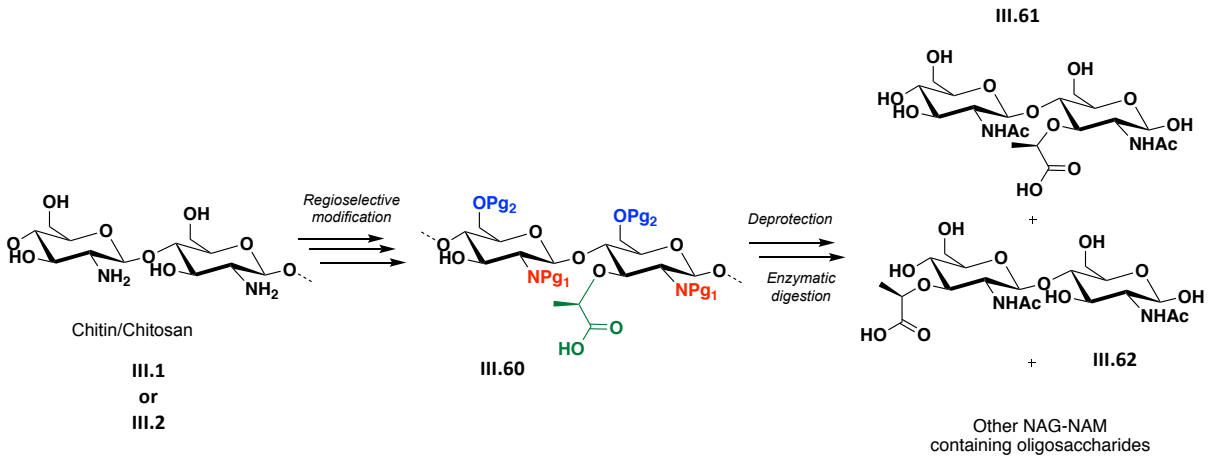


Figure 3.18 Synthetic plan towards NAG-NAM containing oligosaccharides.

3.3 Results and Discussion

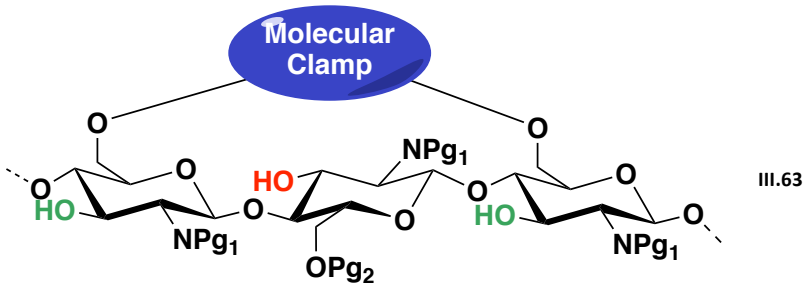


Figure 3.19 Molecular clamp generic representation, highlighting the different O-3 accessibilities.

The most challenging step of the synthetic sequence consisted on the establishment of the conditions for introduction of a lactyl (Lac) moiety at O-3 position of chitosan, in alternate units of glucosamine, Figure 3.19. Chitosan (**1**) was used instead of chitin due to its higher solubility in organic solvents.[166] To ensure this arrangement, a chemical strategy was designed involving a regioselective protection, and the selective attachment of the Lac at a specific O-3 position was attained by regio- and steric discrimination. We anticipated that the natural orientation of the biopolymer[166] would contribute to the differentiation between alternate glucosamine units of a derivative properly functionalized.

Due to the high molecular weight of the biopolymer, the control over the stepwise modification was performed by cross polarization magic angle spinning nuclear magnetic resonance (CP/MAS NMR), infrared (FT-IR) and by ionic liquid chromatography using NAG and NAM as control (see appendix section). However, the line broadening in high molecular weight chitosan derivatives in the NMR spectra, led us to perform the same modifications with chitosan of medium molecular weight. This allowed us to better monitor the chemical modification by CP/MAS NMR.

3.3.1 Chemical modification of chitosan

According to the designed synthetic route, the synthetic sequence started with N-protection of chitosan. The chosen protecting group was the phthaloyl group, Figure 3.20.

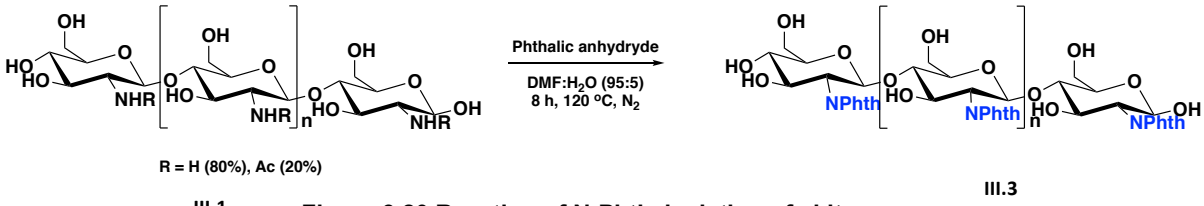


Figure 3.20 Reaction of N-Phthaloylation of chitosan.

Starting with commercially available high molecular weight chitosan (DP = 1800, MW = 293 kDa) (**III.1**) and applying the already reported conditions[171], compound **III.3** was obtained. The use of a mixture DMF:H_2O helps in solubility but also prevents the phthaloyl group from reacting at the O-6 position. Due to the high molecular weight of III.1 the structural analysis had to be done by FT-IR and ^{13}C CP-MAS. The FT-IR analysis confirmed the protection due to the appearance of a double band at 1773 and 1717 cm^{-1}. Also, the ^{13}C CP-MAS analysis confirmed the reaction of N-Phthaloylation due to the appearance of characteristic signal at the aromatic region, 120-140 ppm.

The most challenging step consisted in the establishment of the conditions for the introduction of a Lac moiety at O-3 position in alternate glucosamine units. To ensure this arrangement, a chemical strategy was designed involving a regioselective protection, and the selective attachment of the Lac at a specific O-3 position was attained by regio and steric discrimination, Figure 3.19. We anticipated that the natural orientation of the biopolymer[172] would contribute to the differentiation between alternate glucosamine units of a chitosan derivative properly functionalized. To install the bridges between alternate glucosamines, two clamps **C1** and **C2** were synthesized possessing different lengths, Figure 3.21.

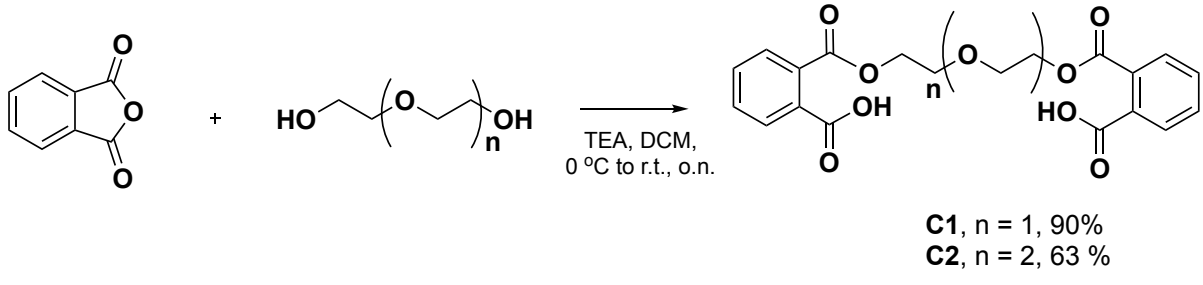


Figure 3.21 Synthesis of the dicarboxylic acids C1 and C2.

The coupling of phthalic anhydride with di- or triethylenoglycol was performed according with the reported procedure.[173] The synthesis of **C1** and **C2** was straightforward leading to its isolation in 90 and 63% yield respectively. The products were confirmed by ^{1}H-NMR and also by HRMS.

Preliminary studies were undertaken to establish the optimized conditions for O-benzoylation such as solvent, reaction time and concentration in order to avoid cross-linking between chains and promote the intramolecular coupling. Different amounts of **C1** and **C2** were tested to maximize the attachment of Lac moiety into compounds **III.64-67** in alternate positions. Thus, treatment of **III.3** with pre-activated acids **C1** or **C2** with CDI in DMF, led to the formation of compounds **III.64-67**, Figure 3.22.

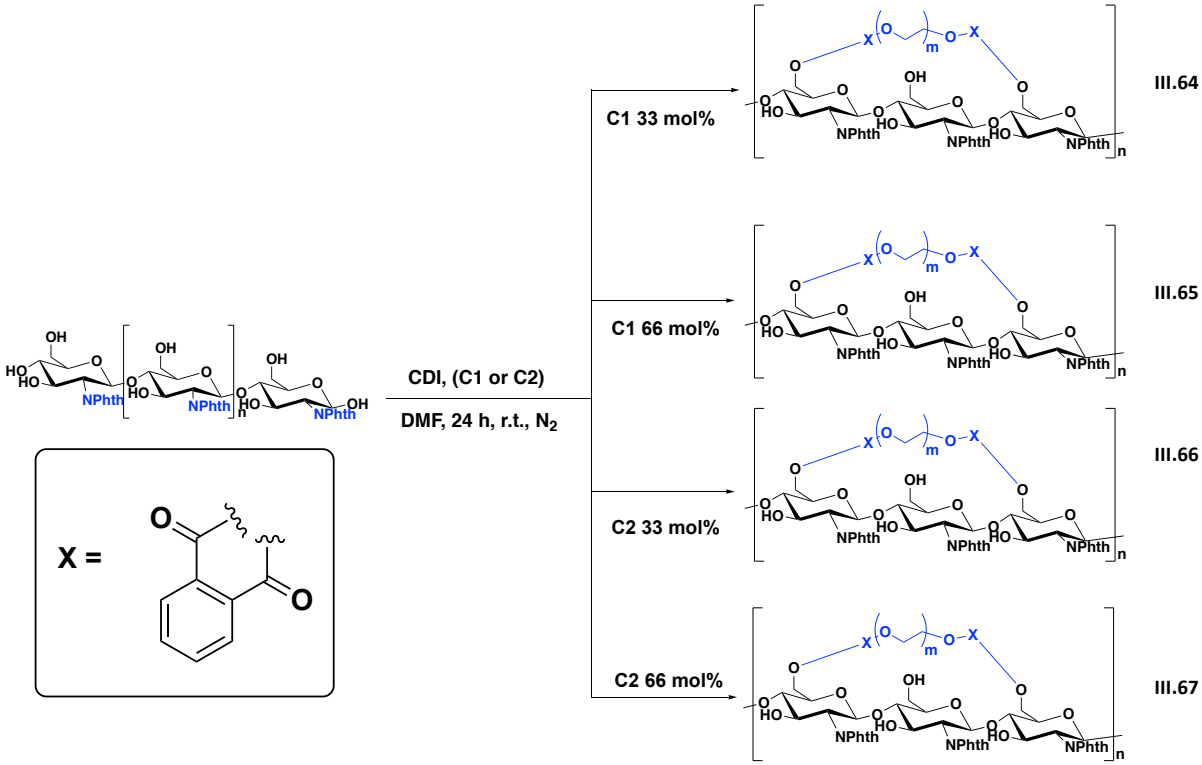


Figure 3.22 Coupling reaction of the dicarboxylic acids to the N-Phth chitosan. C1 m = 1, C2 m = 2

Once again, and due to the polymeric character of the chitosan (**III.1**), the characterization was carried by FT-IR and ^{13}C CP-MAS. At this stage the complexity of the products turned the characterization quite challenging, so a parallel synthesis, with the same reaction conditions, was undertook with a medium molecular weight chitosan (**III.1'**) (DP = 398, MW = 65 kDa). The products obtained by synthetic modification of **III.1'** will be represented with the same

number of those synthetized by modification of **III.1**, adding an apostrophe (i.e. **III.65'** same conditions used to obtain **III.65**). Thus, the products were confirmed by the appearance of a characteristic 1660 cm^{-1} band, ester bond and also the by the number of signals at the aromatic region, 120-140 ppm and also the -OCH_2- groups at 55-70 ppm is clearly perceived, Figure 3.23.

The FT-IR spectra of the product obtained from O-6 benzoylation of **III.3** with the acid C1, compound **III.65**, shows a broad band at 1770 cm^{-1}, indicating the presence of an ester group in both MW substrates, Figure3.23. The ^{13}C CP/MAS spectra, Figure 3.23, shows the prevalence of N-phthaloyl group, but the introduction of the clamp in the high MW substrate, which is observed at 55-70 ppm is not so clear, probably due to the relaxation of this macromolecule. However, in the medium MW substrate **III.65'** the extra resonance due to the -OCH_2- groups at 55-70 ppm is clearly perceived.

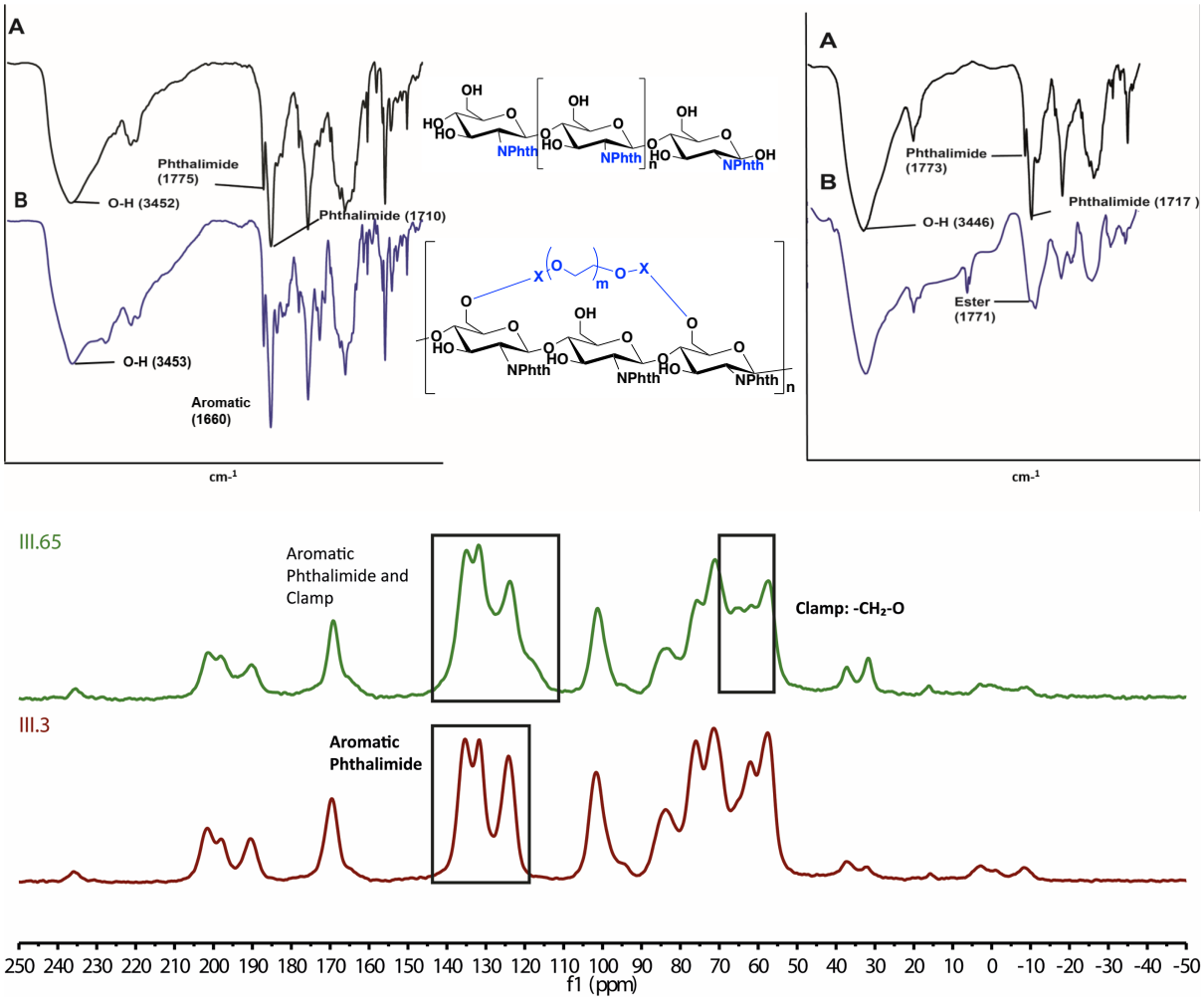


Figure 3.23 FT-IR comparison between III.3-III.3' and III.65-III.65' (medium (left side) and high (right side) molecular weight chitosan); ^{13}C CP-MAS comparison between III.3' and III.65' (medium molecular weight chitosan).

The next synthetic step consisted on the protection of the remaining O-6 positions with TBDMSCl. Thus, compounds **III.64-67** were further silylated with TBDMSCl in the presence of imidazole, in DMF, Figure 3.24.[171]

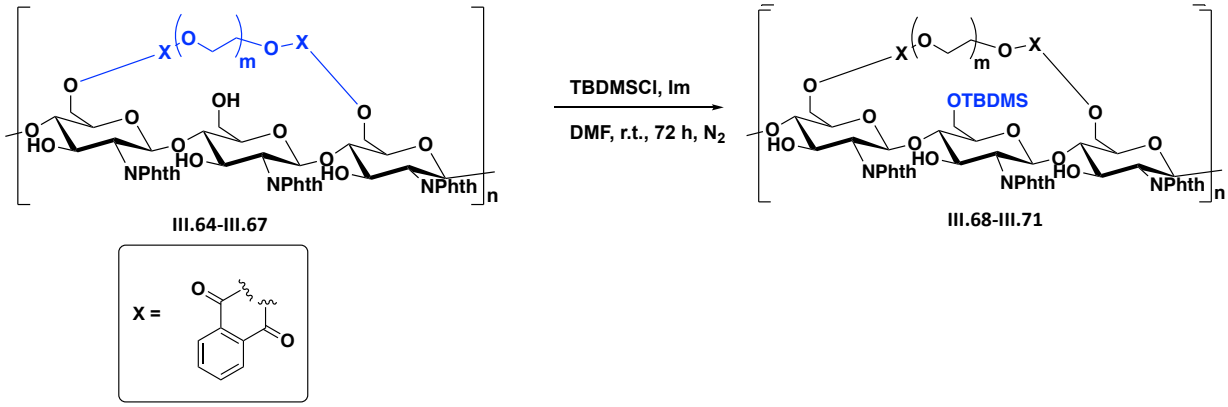


Figure 3.24 Reaction of O-6 Silylation of III.64-III.67.

The O-6 silylation was already described, with a substrate of DS = 1.0. This reaction, Figure 3.24, was performed by treatment of **III.64-III.67** with TBDMSCl, in the presence of imidazole, and the products **III.68**-**III.71** were isolated.

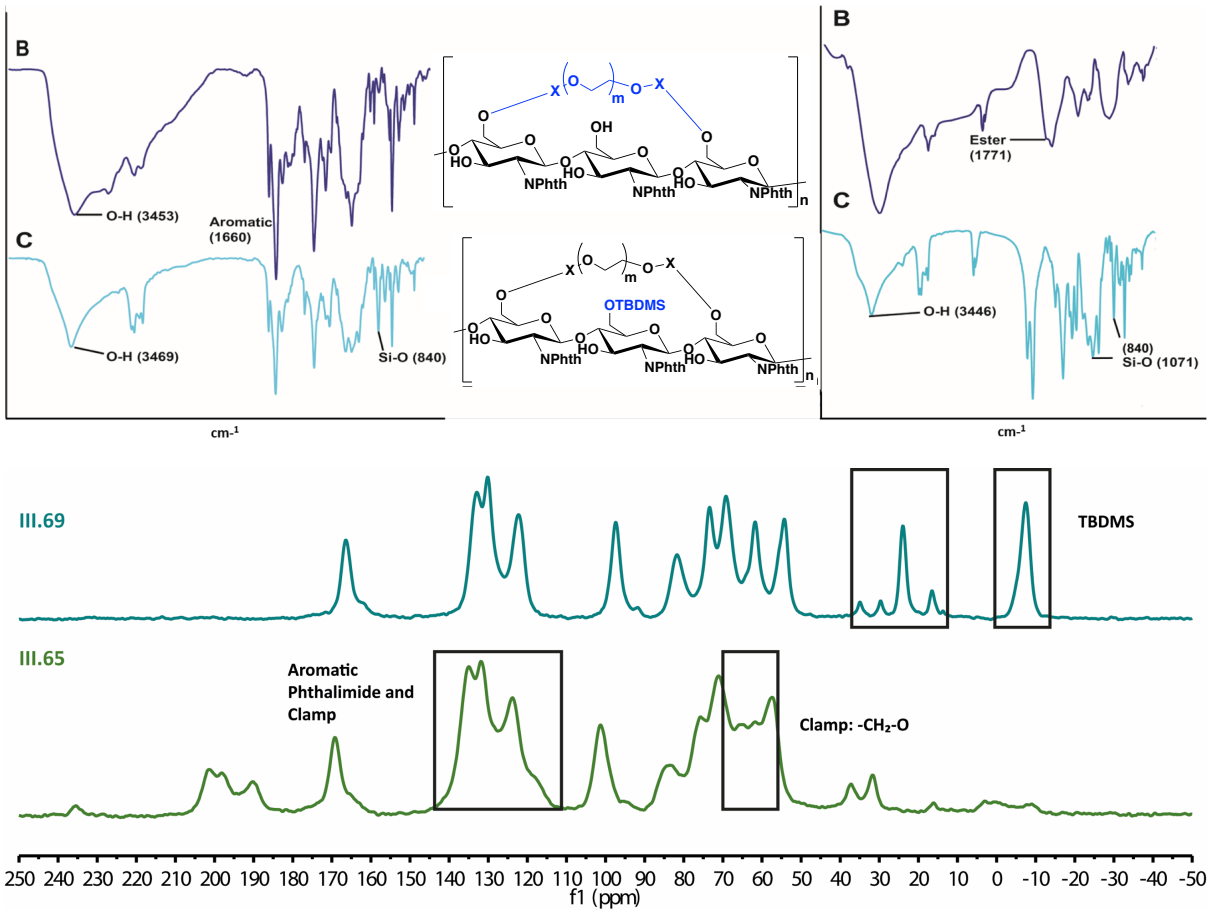


Figure 3.25 FT-IR comparison between III.65-III.65' and III.69-III.69' (medium (left side) and high (right side) molecular weight chitosan); Comparison of the ^{13}C CP-MAS of III.65' and III.69' (medium molecular weight chitosan).

Once again, due to the polymeric character of the compounds the characterization was done by FT-IR and ^{13}C CP-MAS, merging the FT-IR high molecular weight data with the medium molecular weight ^{13}C CP-MAS.

The FT-IR spectra of compound **III.69**, Figure 3.25, shows the appearance of the band at 840 cm^{-1} and the sharpness of the band at 3400 cm^{-1} (O-H bond). In the ^{13}C CP/MAS spectra, the presence of the TBDMS group [171] was confirmed by the presence of the methyl groups at -8, 19 and 25 ppm, Figure 3.25, which were not present at the starting material (**III.65'**). The remaining products showed similar spectra profiles. The transformations performed on the medium MW chitosan **III.65'** allowed a clear identification of the structural modifications with this synthetic procedure.

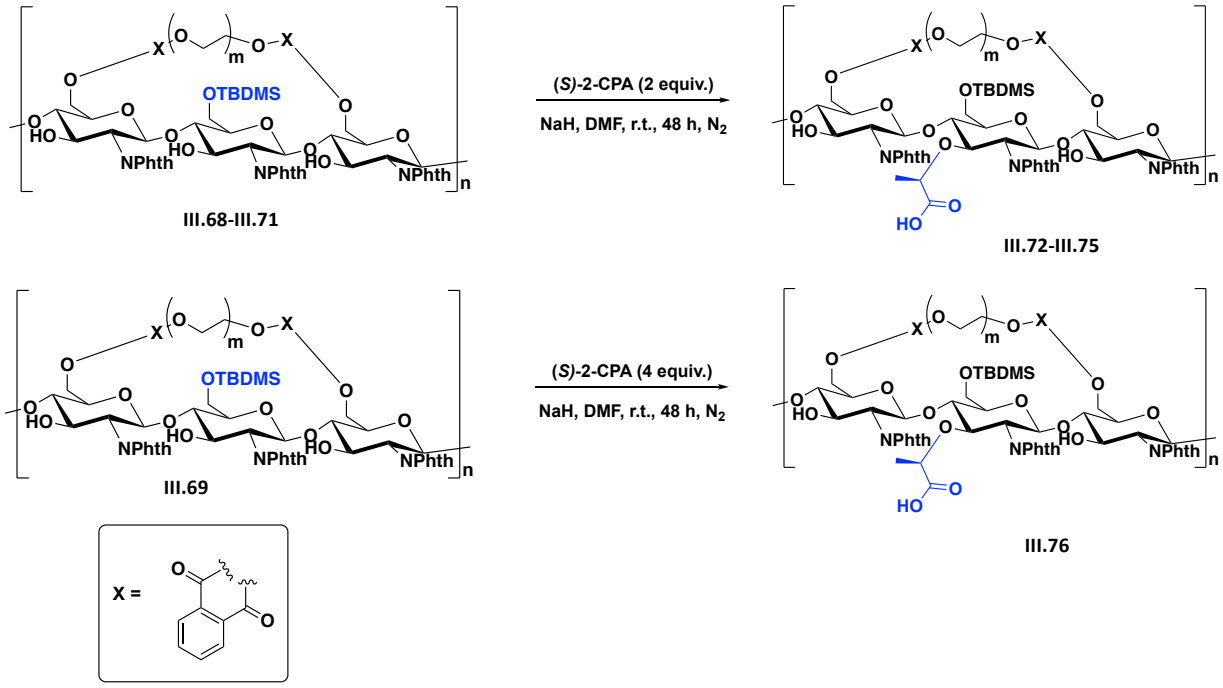


Figure 3.26 Lactyl moiety insertion reaction scheme.

At this stage, compounds **III.68**-**III.71** were protected at positions N-2 and O-6, and the O-3 position remains free. It was then envisaged to introduce the Lac group taking advantage of the asymmetry created by the molecular clamp strategy. Two distinct groups reagents were tested to insert Lac at O-3 position: ethyl (*S*)-(–)-2-(trifluoromethylsulfonyloxy)propionate and (*S*)-(–)-2-chloroprionic acid (2-CPA).

The ethyl (*S*)-(–)-2-(trifluoromethylsulfonyloxy)propionate has been used by us and others to install the Lac moiety at O-3 of glucosamine drivatives *See Section 2.4.1*). However, the preliminary studies showed that this reaction did not give a good DS (DS = 0.1). So, 2-CPA was used instead, Figure 3.26.

The aim was to obtain NAG-NAM containing oligosaccharides after enzymatic hydrolysis with a ratio of NAG/NAM similar to that found in bacteria such as *E. coli* or *S. aureus*, that have a 1:1 to 1:1.2 ratio, respectively. Thus, the DS should be 0.5-0.6, if the final product that we have obtained was a mimetic of *E. coli* or *S. aureus* PGN.

After deprotonation with NaH the 2-CPA was used. Two loadings of 2-CPA were used, 2 equiv. or 4 equiv. (*per* glucosamine unit). All the starting materials (**III.68**-**III.71**) were reacted with the 2 equiv. lof 2-CPA to afford **III.72**-**III.75** and only **III.69** reacted with 4 equiv. of 2-CPA to afford **III.76**. After isolation, the products **III.72**-**III.76** were characterized by FT-IR and ^{13}C CP-MAS. The FT-IR shows a 1643 cm^{-1} band, corresponding to the carboxylic acid (C=O) and also a wide band at 3422 cm-1 correspondent to the free OH from the carboxylic acid.

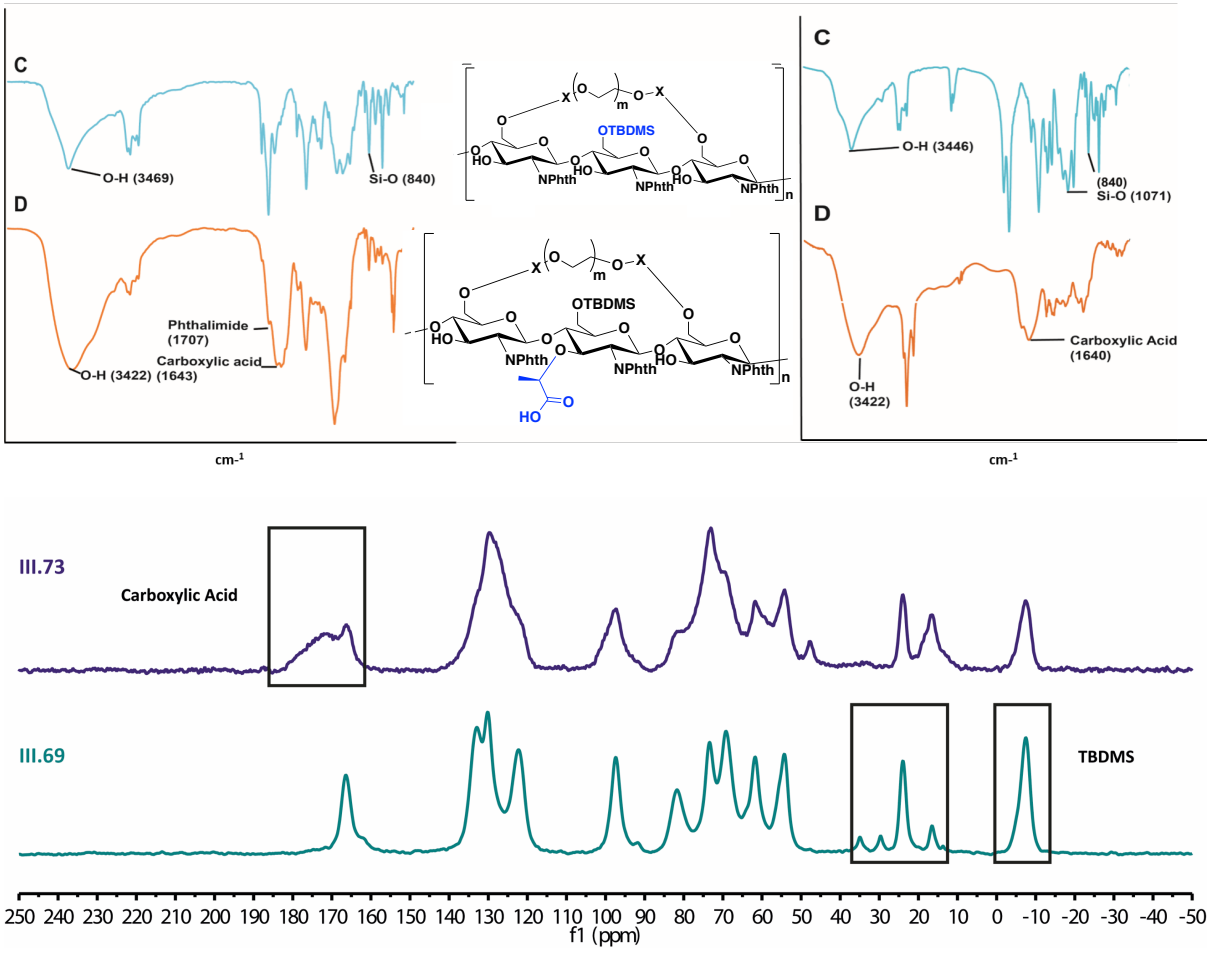


Figure 3.27 FT-IR comparison between III.69-III.69' and III.73-III.73' (medium (left side) and high (right side) molecular weight chitosan); Comparison of the ^{13}C CP-MAS of III.69 and III.73 (medium molecular weight chitosan).

The ^{13}C CP-MAS spectra shows the appearance of a signal at 180 ppm corresponding to the carbonyl group, from the carboxylic acid, and also a modification in the 70-80 ppm region due to the Lac methyne group. The correct placement of Lac unit at O-3 was evaluated in Section 3.22-3.24.

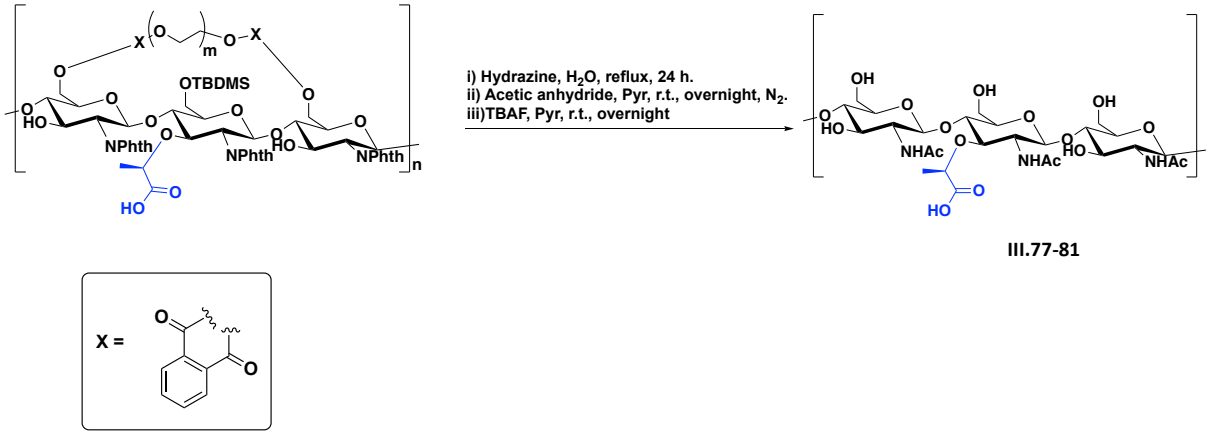


Figure 3.28 Synthetic sequence for removal of the protecting groups and N-acetylation.

The compounds **III.72**-**III.76** possess all the required structural functionalities needed to mimic the PGN sugar backbone: lactyl unit at position O-3; the β-(1,4) glycosydic bond and the N-acetyl group. The last steps consisted on sequential removal of the phthalimide group with simultaneous removal of the ester clamp, by treatment with hydrazine solution, under reflux conditions for 24 hours. After solvent evaporation the residue was washed with water and ethanol.

N-acetylation was performed by reaction with acetic anhydride in dry pyridine in an ice-cold bath for 16 hours. The solvent was evaporated, and the residue was dissolved in water and the pH adjusted to 3 precipitating the product. To afford the N-Acetylated compounds, and finally silyl groups removal, was achieved by treatment with a 1 M TBAF solution for 16 hours, and afforded compounds **III.77-81**.

The FT-IR spectra of product **III.79** obtained after removal of the protecting groups and N-acetylation, showed the presence of the free O-H (3445 cm^{-1}), the amide group (1698 cm^{-1}) and a carboxylic acid group (1656 cm^{-1}), indicating that all protecting groups have been successfully removed, Figure 3.29E.

In Figure 3.29 is also showen a ^{13}C CP-MAS comparison between High Molecular Weight and Medium Molecular Weight chitosan

Figure 3.30, summarizes the synthetic plan towards the products **III.77-81**.

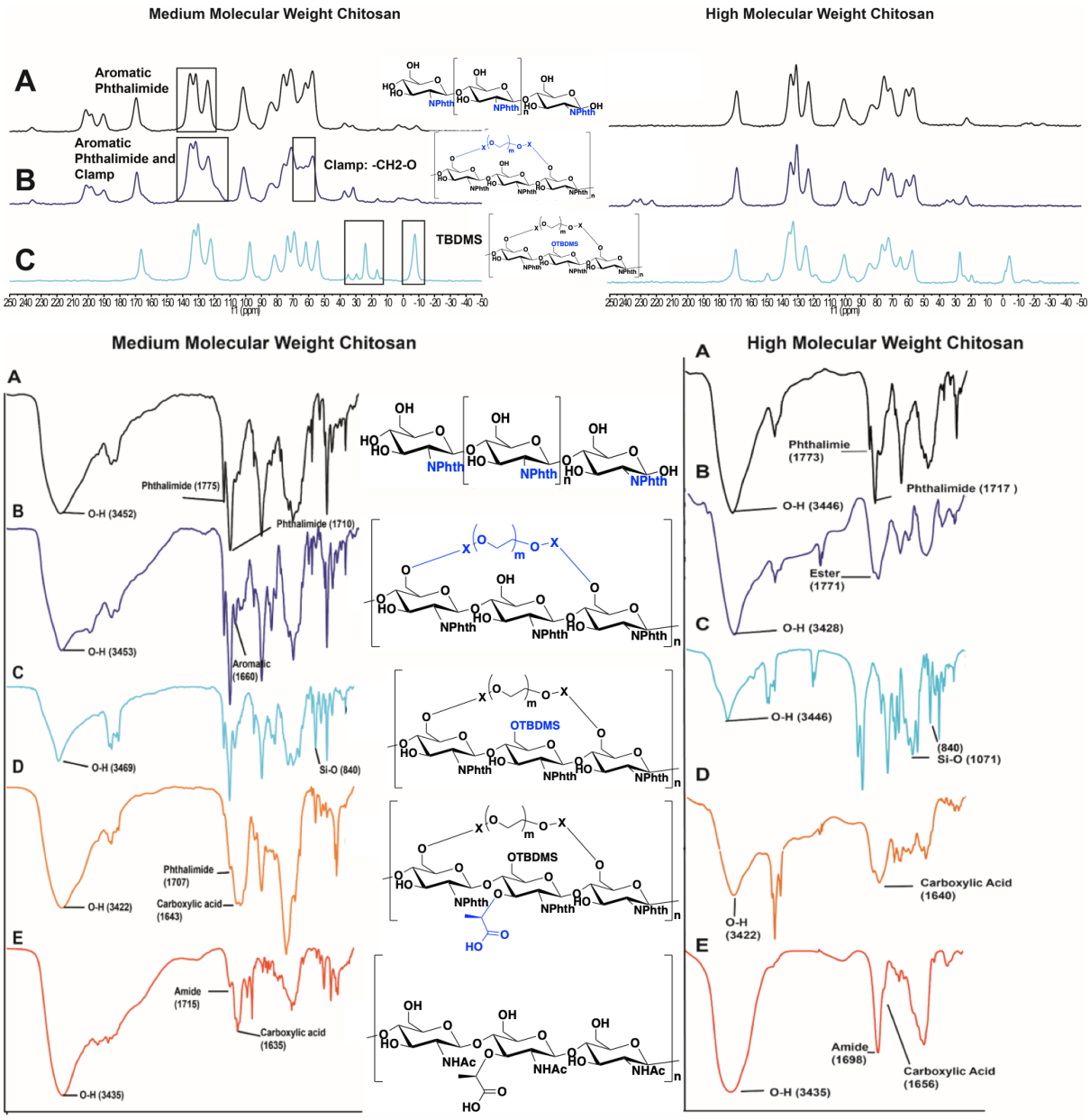


Figure 3.29 Comparison of the ^{13}C CP-MAS and FT-IR comparison of medium (left side) and high (right side) molecular weight chitosan along the synthetic sequence.

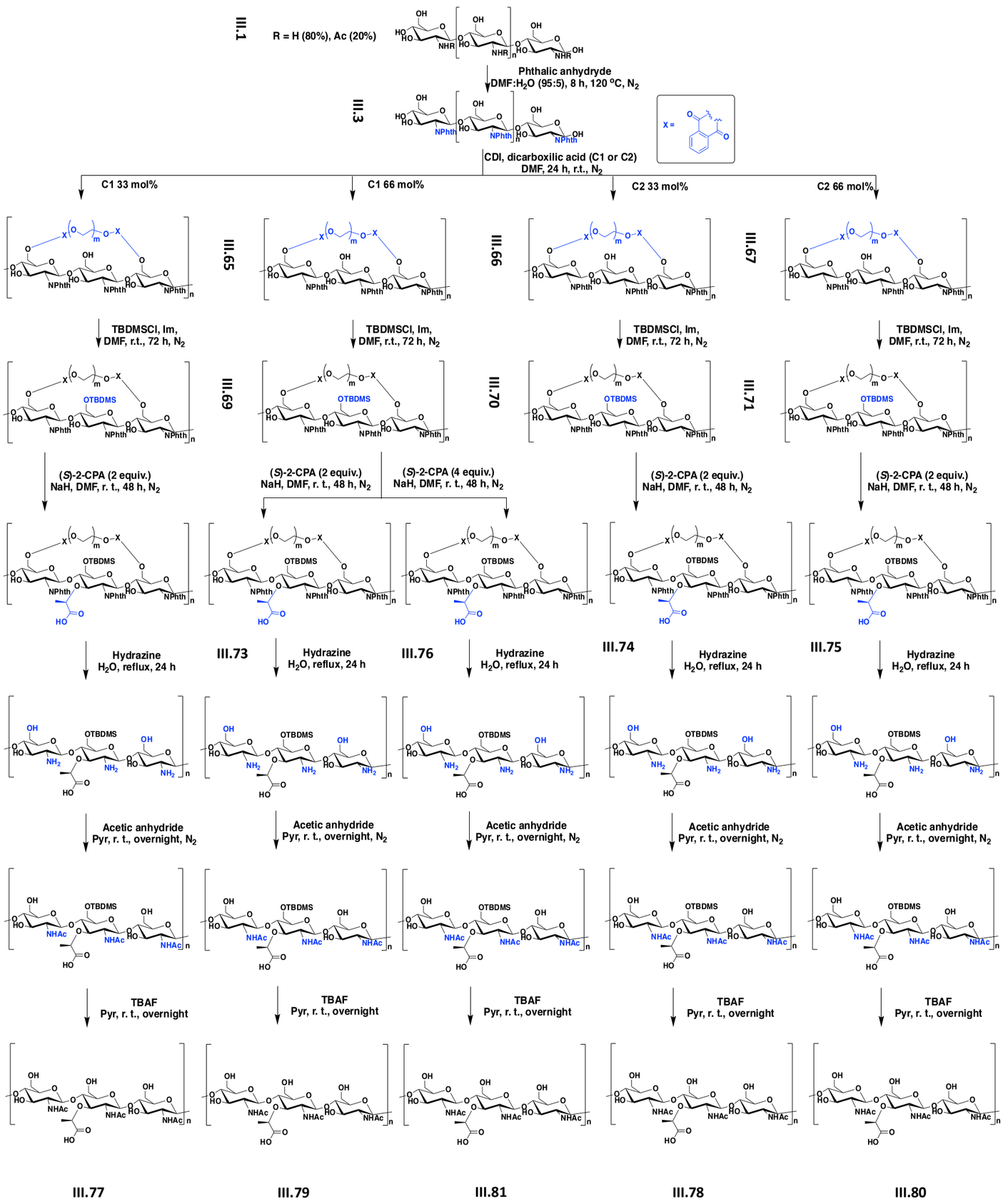
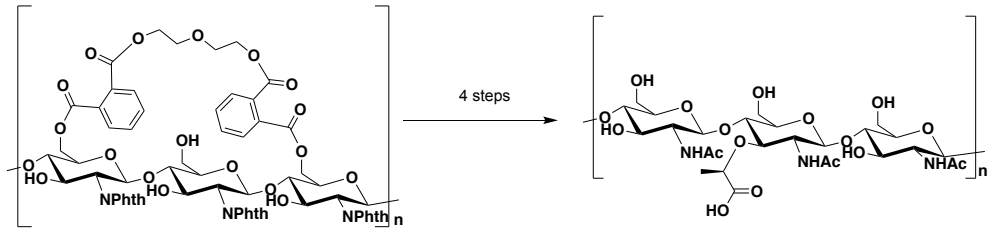


Figure 3.30 Synthesis summary to afford the products II.77-81

After structural characterization, and in order to evaluate the amount of lactate moiety successfully installed, we needed to determine the ratio of NAM and NAG moieties. This was achieved when compounds **III.77-81** were hydrolyzed with HCl 3 M for 3 h at 95 ^{o}C, followed by ionic liquid chromatography (using a Dionex system), (Table 3.2).

The ratio NAM/NAG of bacterial PGN from *Staphylococcus aureus* and *Escherichia coli* by chromatography is 1.2 and 1, respectively. Purified PGNs from these bacteria were used as positive controls (Table 3.2, entries 6 and 7).

Table 3.2 NAM:NAG ratio obtained by different synthetic strategies.



III.64 – III.67 **III.77 – III.81**

Entry	Sample	Bridge (mol%)	2-CPA (equiv)	**Ratio[a] NAM/NAG**
1	**III.77**	**C1** (33)	2	**0.95**
2	**III.78**	**C2** (33)	2	**1.52**
3	**III.79**	**C1** (66)	2	**1.21**
4	**III.80**	**C2** (66)	2	**1.38**
5	**III.81**	**C1** (66)	4	**1.85**
6	*S. aureus*	__	__	**1.2**
7	*E. coli*	__	__	**1**

[*i*] 1) TBDMSCl, imidazole, DMF, N_2, r.t., 72 h; 2) (S)-2-CPA (2 or 4 equiv.), NaH, DMF, r.t., 48 h; 3) i) NH_2NH_2, H_2O, reflux, 24 h; ii) Ac_2O, pyridine, r.t., overnight; 4) TBAF, pyridine, r.t., overnight; [a] Measured by HPAEC-PAD using in a Dionex ICS-5000 system with a CarboPac PA10 column. Eluted with an isocratic step at a 18 mM NaOH for the first 20 min., followed by a gradient step from 20 to 25 min. of 0 to 0.2 M of sodium acetate, followed by a isocratic step from 25 to 30 min. at 0.2 M of acetate and a final gradient step from 30 to 35 min. from 0.2 to 0.8 M of acetate to wash the column

First experiments were carried with both dicarboxylic acids **C1** and **C2** at 33% mol ratio with monomer unit of **III.3** and 2 equivalents of (S)-2-CPA (Table 3.2, entries 1 and 2). Compound **III.77**, prepared with **C1** had a significant but lower NAM/NAG ratio (0.95) than those obtained with bacterial samples. In order to modulate the ratio NAM/NAG, a higher amount of the dicarboxylic acid was also used (Table 3.2, compounds **III.79** and **III.80**, entries 3 and 4). This resulted in a higher formation of NAM as it can be seen in compound **III.79** (1.21), which results from the use of the shorter bridge **C1** at 66 mol%. Similar result was obtained for compound **III.80** (1.38), which results from the use of longer bridge **C2**. An increased amount

of (S)-2-CPA also led to a higher amount of NAM as expected (compound **III.81** (1.85)). The ratio of NAM/NAG indicated a significant incorporation of NAM residues on the chitosan skeleton but gave no clue about how alternate the NAM units had been installed.

3.3.2 Biological evaluation of the PGN mimetics

Drosophila flies recognize the presence of PGN with the help of specific PGN receptors: PGRPs (PGN recognition proteins). Depending on the composition of the injected PGN and the PGRP receptor involved, one of the two distinct signaling pathways is activated to produce antimicrobial peptides capable of killing the infected pathogen.

In flies, the Toll pathway is required for resistance to Gram-positive bacteria, containing a lysine-type PGN, as well as fungi and yeast pathogens. The Toll receptor is activated when bacterial lysine-type PGN is recognized by PGRPs, specifically PGRP-SA and PGRP-SD, as well as Gram-negative binding proteins (GNBPs).[174] Then, proteolytic cascades, which are not yet well characterized, lead to activation of Spätzle-processing enzyme (SPE) that leads to the Spätzle cleavage in the hemolymph. The processed Spätzle binds as a dimer to Toll leading to its dimerization in the plasma membrane leading to activation of three proteins Myd88, Tube and Pell and recruiting to the Toll receptor in the cytoplasmatic region. Subsequently, Cactus is phosphorylated and degraded by proteasome. Transcription factors Dif and Dorsal are released and moved through the nucleus and then activate the transcription genes that encode several antimicrobial peptides leading to the immune response.[175]

The Imd pathway is directly associated to activation by Gram-positive bacteria, possessing diaminopimelic acid-type PGN (DAP-PGN). The Imd pathway is activated by recognition of monomeric DAP-PGN by PGRP-LC/LE, then Imd interacts with dFADD which binds the apical caspase Dredd, enhancing the cell death activity and proteolytic processing the Dredd.[176] At the same time a cascade is activated leading to the phosphorylation of IKK signaling complex which also phospholyrate the Relish. This leads to its cleavage and the Rel domain translocate to the nucleus, where it activates the transcription genes that encode several antimicrobial peptides leading to the immune response.[176-179]

Despite all the studies developed to understand innate immune response and the recognition process between host and bacteria during bacterial infection, some questions remain unanswered. How host receptors can recognize high molecular weight fragments of PGN at

the surface of bacteria remains unsolved. It is also not clear which are the structural requirements for PGN recognition by PGRPs.

Thus, in order to investigate if the sample **III.79**, which provided the closest NAM/NAG ratio compared to *S. aureus*, would be recognized by PGRP-SA, PGRP pull-down assays were performed using mCherry-PGRP-SA, a N-terminal fluorescent tagged protein derivate from *D. melanogaster* flies.

In this study, sample **III.79** was subjected to two distinct experiments in the presence and absence of the natural ligand PGN extracted from *S. aureus*.

In Figure 3.31 it is possible to compare the binding affinity of mCherry_PGRP-SA (PGRP) to sample **III.79** and to PGN, by pull-down assays. The amount of PGRP that is pulled down to the pellet with an incubation with only PGN, is shown in lane 2. In lane 3, it is possible to observe an increase on the amount of PGRP that is pulled down when PGRP was incubated with both PGN and sample **III.79**. This result suggests that the PGRP has affinity for this sample. In order to clarify this result, the same experiment was performed in the absence of natural PGN (lane 5 and lane 6). In lane 6 (PGRP incubated only with **III.79**) it was observed an increase in PGRP amount present in the pellet. This indicates that PGRP has some affinity to sample **III.79**.

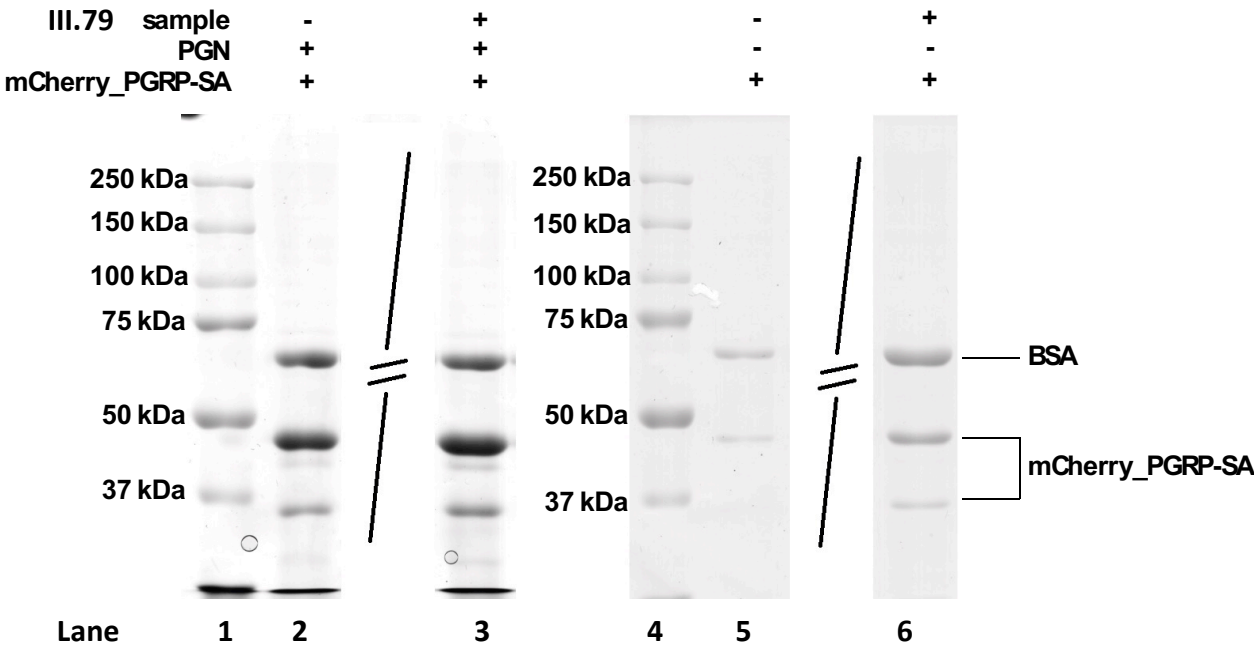


Figure 3.31 Binding affinity of mCherry_PGRP-SA to sample III.79 and purified PGN from *S. aureus* NCTC 8325-4 by pull down assay. BSA is added as a loading control to all samples. Ran on 8% SDS-PAGE gel.

These results show that sample **III.79** is similar to the sugar backbone of PGN. However, since the use of BSA as internal loading control did not have the same intensity in both lanes other enzymatic recognition experiments were explored in order to understand how similar it was. In order to evaluate alternation of NAM residues, samples **III.77-81** were further digested with hydrolytic enzymes and run on HPLC.

3.3.3 Enzymatic assays

Therefore, and regarding the previously PGRP and Dionex results, we envisaged new enzymatic recognition experiments, using mutanolysin and lysozyme as lytic enzymes for the natural PGN, with the objective to understand if our **III.77-81** sample could be recognized/hydrolysed by them. In fact, these experiments would give us an idea of the NAG-NAM composition along the polysaccharide structure.

Several conditions were tested in order to optimize the HPLC profile.[180] Digestion of PGN of Several conditions were tested in order to optimize the HPLC profile.[180] Digestion of PGN of *E. coli* by mutanolysin and by lysozyme was performed (*Appendix Section Figure S2*), although the peaks from the peptide stems complicate the chromatogram analysis. Compound **III.79** gave the best hydrolysis profile on HPLC when digested either by mutanolysin or by lysozyme, Figure 3.32, where it can be seen several peaks between 7 and 15 minutes that are generated by enzymatic hydrolysis, since they are not presence on the control samples. Additionally, the ratio NAM/NAG (1.21) obtained for **III.79** by Dionex was also the most similar to the bacterial ratio of *S. aureus*, Table 4.2.

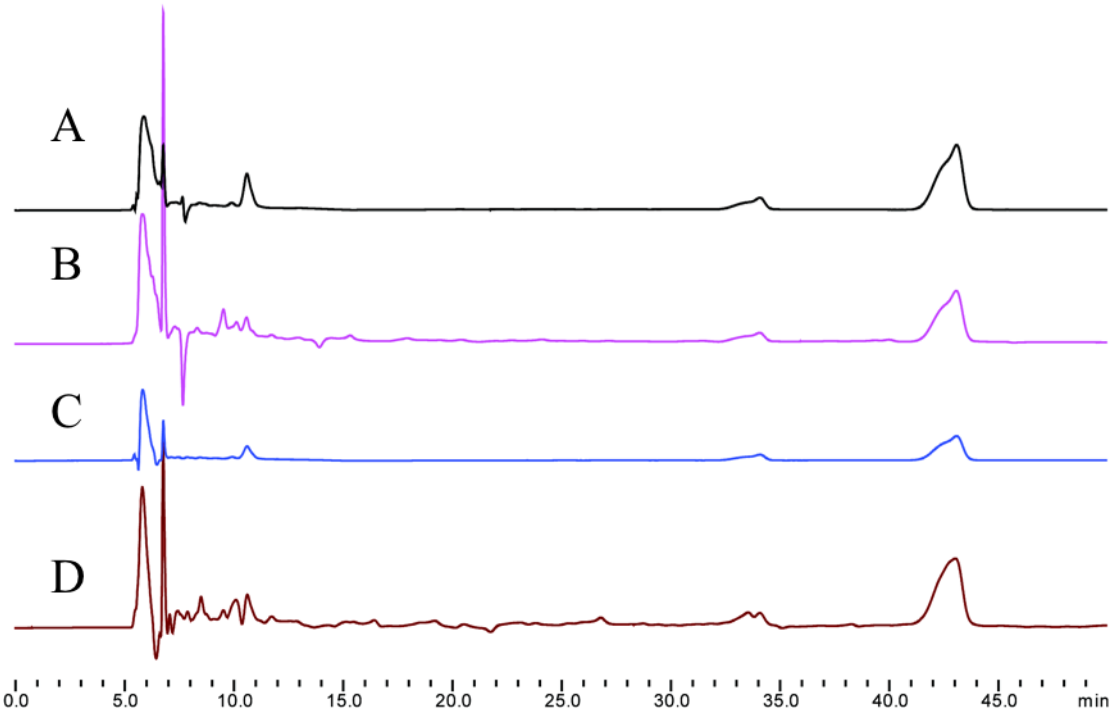


Figure 3.32 A – Lysozyme negative control (under the same conditions: III.79, buffer, temperature and reaction time without enzyme) – black; B – Lysozyme digestion – purple; C – Mutanolysin negative control (under the same conditions: III.79, buffer, temperature and reaction.

As shown in Figure 3.32, the digestion of **III.79** by lysozyme, Figure 3.32B, as well as mutanolysin, Figure 3.32D, generates several fragments (containing NAG-NAM moieties) in the region 7 to 12.5 min, characteristic of the PGN carbohydrate digestion by the same enzymes.[180] Moreover, the weak recognition of samples **III.77**, **III.78**, **III.80** and **III.81** by these enzymes indicates that the oligosaccharides composition does not mimic the PGN carbohydrate backbone despite of the NAG/NAM ratio determined by the Dionex experiments.

Despite all the efforts in order to collect the peaks generated after enzymatic hydrolysis we were not able to collect and desalt it to perform liquid state NMR. In order to clarify the composition of the fragments released during the enzymatic digestion a LC-MS were performed in order to analyze the mass of it.

3.3.4 LC-MS experiments

This part of the work was made in collaboration with Prof. Maria do Bronze at Faculdade de Farmácia da Universidade de Lisboa. The HPLC results from enzymatic digestion of compounds **III.77**, **III.78**, **III.80** and **III.81** (*Appendix Section Figure S1-7*) (lysozyme and

mutanolysin) demonstrate the relative position of NAM is not as alternated as in **III.79**. Indeed, a less efficient digestion was observed for the compounds **III.77**, **III.78**, **III.80** and **III.81**. The enzymatic digestion revealed that alternating NAM formation on chitosan backbone was obtained when a higher loading of a shorter and less flexible bridge **C1** is used. An experiment control was performed: chitosan N-phthaloylation and O-silylation, in the absence of a clamp, followed by Lac introduction. The Dionex analysis showed a low NAM/NAG ratio, supporting the important role of the use of an appropriate clamp. An experiment control was performed: chitosan N-phthaloylation and O-silylation, in the absence of a clamp, followed by lactyl introduction. The Dionex analysis showed a low NAM/NAG ratio, supporting the important role of the use of an appropriate clamp.

We next evaluated the composition of the fragments released by digestion with mutanolysin by LC-MS/MS analysis. Mutanolysin only recognizes NAG-NAM sequences,[160] hydrolysing the glycosidic bond between NAM and NAG (does not hydrolyze chitin (*Appendix Section Figure S10*). The m/z 499 ($[NAG\text{-}NAM+H]^+$ or $[NAM\text{-}NAG+H]^+$) and m/z 521 ($[NAG\text{-}NAM+Na]^+$ or $[NAM\text{-}NAG+Na]^+$) were selected to have a quick elucidation for the target disaccharide, Figure 3.33 A and B. The data obtained showed that the $[NAG\text{-}NAM+Na]^+$ disaccharide is present with a retention time of 9.5 min (*Appendix Section Figure S8 and S10*). The mass spectra of LC-MS/MS of disaccharide released from E. coli peptidoglycan and sample **III.79** showed a similar fragmentation pattern (*Appendix Section Figure S11*). However, the most intense fragment detected on the LC-MS was observed at 10.48 min, with m/z of 724, which corresponds to the trisaccharide ($NAG\text{-}NAG\text{-}NAM+Na^+$) (Figure 3.33C and *Appendix Section Figure S10*). From the TCI it was possible to determine the percentage of digested polymer, and it was found that 55% of the initial **III.79** sample was solubilized by mutanolysin (*see Appendix Section*). A broad peak was found from 35 to 55 min with 870 m/z, corresponding to undigested polymer. Quantification studies performed by mass spectrometry indicated that from the 55% digested sample **III.79**, 33% contained NAG-NAM oligosaccharides: composed of 7% of disaccharide NAG-NAM and 26% of NAG-NAG-NAM (in separated peaks) (*Appendix Section Figure S10*).

The LC-MS/MS (*Appendix Section Figure S11*) analysis combined with the data from the ionic chromatography suggests that the strategy applied favored the 2-CPA insertion at every third glucosamine unit, distinguishing two adjacent glucosamine units. Thus, compound **III.69** (with **C1**) is converted in a derivative possessing NAG-NAM-NAG oligosaccharides which are

recognized by mutanolysin originating the trisaccharide NAG-NAG-NAM as major fragment along with the NAG-NAM disaccharide.

Further studies were attempted to improve the method into a multigram scale (3 g). However, our results revealed that due to the polymer character of intermediates a one-gram scale allows superior control over the designed alternate structural modification.

In a reference reaction starting from 300 mg of chitosan, 230 mg of **III.79** were obtained. After the enzymatic digestion of a 1 mg aliquot, 0.55 mg were hydrolysed (measured by TCI analysis using as calibration curve a disaccharide NAG-NAM synthesized according a previous procedure[181]). Of these solubilized materials, by means of HPLC-TCI quantification it could be deduced that 26% correspond to NAG-NAG-NAM trisaccharide and 7% to NAG-NAM disaccharide. Other peaks in the chromatogram with long retention times contained longer oligosaccharides richest in NAM and less susceptible to the total enzymatic hydrolysis.

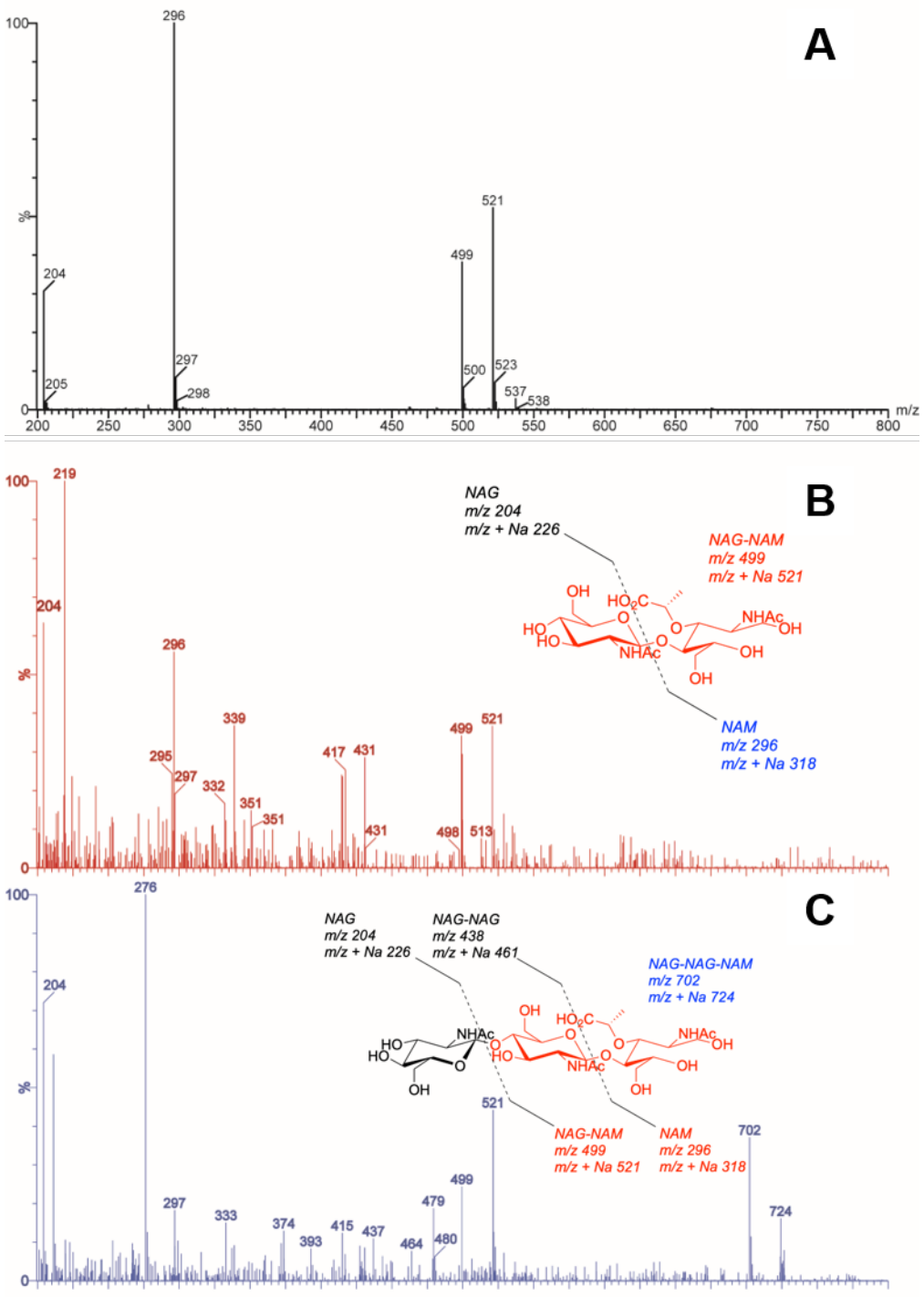


Figure 3.33 LC-MS spectra at m/z 521: E. coli (A) and III.79 at 9.52 min (B); MS spectra from sample III.79 at 10.48 min (C).

3.4 Conclusions

In this chapter it is reported approaches towards the synthesis of PGN fragments using as starting material chitosan (**III.1**).

It was developed an innovative, fast and simple strategy for the synthesis of NAG-NAM containing oligosaccharides, based on a combined synthetic and enzymatic route. With the choice of a proper molecular bridge and reaction conditions, it is possible to convert chitosan into valuable compounds that have been prepared by long multistep procedures. The oligosaccharides produced **III.79** consist of surrogates of the carbohydrate backbone of bacterial cell wall PGN, that once hydrolyzed by lytic enzymes release shorter oligosaccharides containing NAG-NAM units. The surrogates produced are recognized by important recognition systems associated with bacterial infections, including the PGN recognition receptor from *Staphylococcus aureus* (PGRP-SA). This strategy provides a significantly cheaper and fast route to achieve fragments of the carbohydrate backbone of PGN. The methodology we have presented can be applied to commercial chitosan (**III.1**) and used to formation of NAG-NAM containing oligosaccharides after enzymatic digestion and separation by column chromatography with a 33% yield. The compounds generated by this approach can be further explored on the assembly of bacterial PGN and used to investigate the recognition of bacteria by different hosts. Similar strategies can be investigated for the synthesis of other carbohydrates.

3.5 Experimental

All commercially obtained reagents were used without further purification unless specified. All the mentioned solvents used in the reactions were dried by usual methods. Molecular sieves 4Å were activated by heating at 300 °C for 3 h. Preparative and analytical TLC was performed with silica gel 60 plates of 1 mm, 0.5 mm and 0.25 mm, respectively. Nuclear Magnetic Resonance (NMR) spectra were recorded at Bruker Advance 400 MHz for ^{1}H and at 100 MHz for ^{13}C, in $CDCl_3$, DMSO-d_6 or D_2O with chemical shift values (δ) in ppm downfield from TMS (0 ppm) or the solvent residual peak of D_2O (4.79 ppm), DMSO-d_6 (2.50 ppm) or $CDCl_3$ (7.24 ppm) as internal standard. The chemical shifts (δ) for proton spectra were expressed in parts per million (ppm) and the data obtained was presented in the following order: deuterated solvent, signal chemical shift (δ), relative intensity, spin multiplicity (s – singlet, d – doublet, t – triplet, m – multiplet, dd – doublet of duplets), coupling constant (J, in Hz) and molecule peak attribution if possible. The data for carbon spectra was presented in the following order: solvent, chemical shift (δ), molecule attribution if possible. ^{13}C CP/MAS NMR spectra were recorded both at 100.62 MHz on a Bruker Avance III 400MHz (9.4 T) spectrometer using the following parameters: time between scans(D1): 3 seconds, Channel 1H (90 deg. pulse), (P3) pulse length: 3 microseconds (PL12), power level: 6.94 dB, Channel ^{13}C (P15) CP contact time: 2000 microseconds, (PL1) CP power level: 9.6 dB, 15 kHz spinning rate; and at 75.47 MHz on a Bruker Avance III 300MHz (7.2 T) spectrometer using the following parameters: time between scans (D1): 10 seconds, Channel 1H (90 deg. pulse) (P3) pulse length: 4 microseconds, (PL12) power level: -17.68 dB Channel ^{13}C (P15) CP contact time: 1200 microseconds, (PL1) CP power level: -20.79 dB, and 5 kHz spinning rate. Infrared (IR) spectra were recorded on a Bruker Tensor 27 spectrophotometer FTIR spectra were recorded on Perkin-Elmer Spectrum 1000 model apparatus in KBr dispersions for solid samples or NaCl dispersions for oil samples. In each spectra description only the more intense and characteristic bands were identified. The data obtained is presented in the following order: sample support (NaCl or KBr); frequency of the maximum absorption band (ν_{max} in cm^{-1}attribution to a functional group in a molecule if possible.

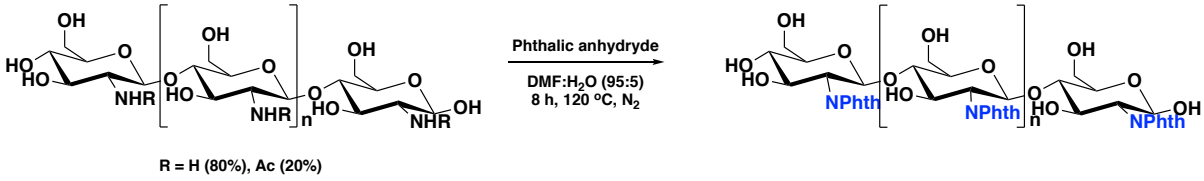
The reactions were followed by thin layer chromatography (TLC) silica gel 60 G/UV254 Macherey-Nagel with 0.20 mm. Spots detection on TLC was carried with UV light using a 254

nm lamp (Vilber-Lourmat). Additionally, TLC plates visualization was carried with a TLC spray solution of ethanol-sulfuric acid 9:1.

Chitosan (**1**) 80+ high molecular weight (MW = 393 kDa) was purchased from AltaKitin. Chitosan (**1'**) 80+ medium molecular weight (MW = 68 kDa) was purchased from AltaKitin.Mutanolysin from *Streptomyces globisporus* was purchased from Sigma Aldrich (≥4,000 units/mg protein). Lysozyme from egg white was purchased from Sigma Aldrich (≥40,000 units/mg protein).

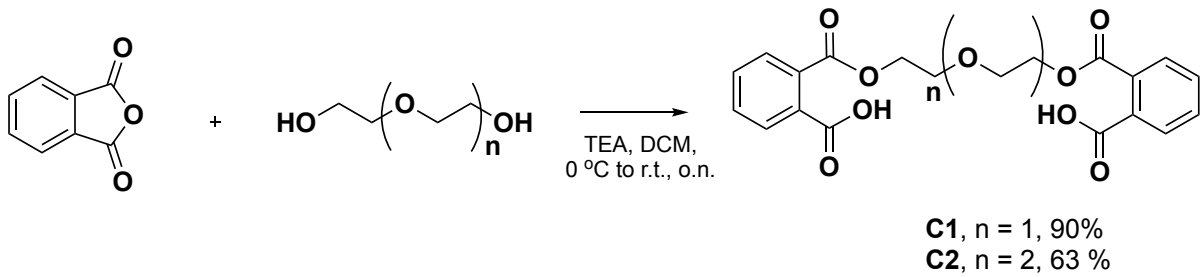
3.5.1 Synthesis

3.5.1.1 Preparation of N-phthaloyl chitosan (III.3)



Preparation of N-phthaloyl chitosan (**III.3**): To a solution of 5.6 mmol of the anhydride – phthalic or di-phenyl maleic – in DMF (6 mL) containing 5% of water was added chitosan (**III.1**) (300 mg, 1.89 mmol), and the mixture was heated overnight under a nitrogen atmosphere at 120 ºC with stirring. The reaction mixture was cooled and then poured into ice/water (100 mL). The precipitate was collected on a filter, washed with 150 mL of methanol for 1 h and dried to give the product **III.3** as a brown solid (400 mg) in 72% yield. **^{13}C CP/MAS NMR** (101 MHz): δ 168.8 (C=O), 134.5, 130.9, 123.1 (Ar), 100.6 (C1), 82.3 (C4), 75.0 (C5), 71.0 (C3), 60.9 (C6), 56.3 (C2). **FT-IR** ν_{max} (KBr): 3446, 2928, 1773, 1717, 1641, 1196-990.

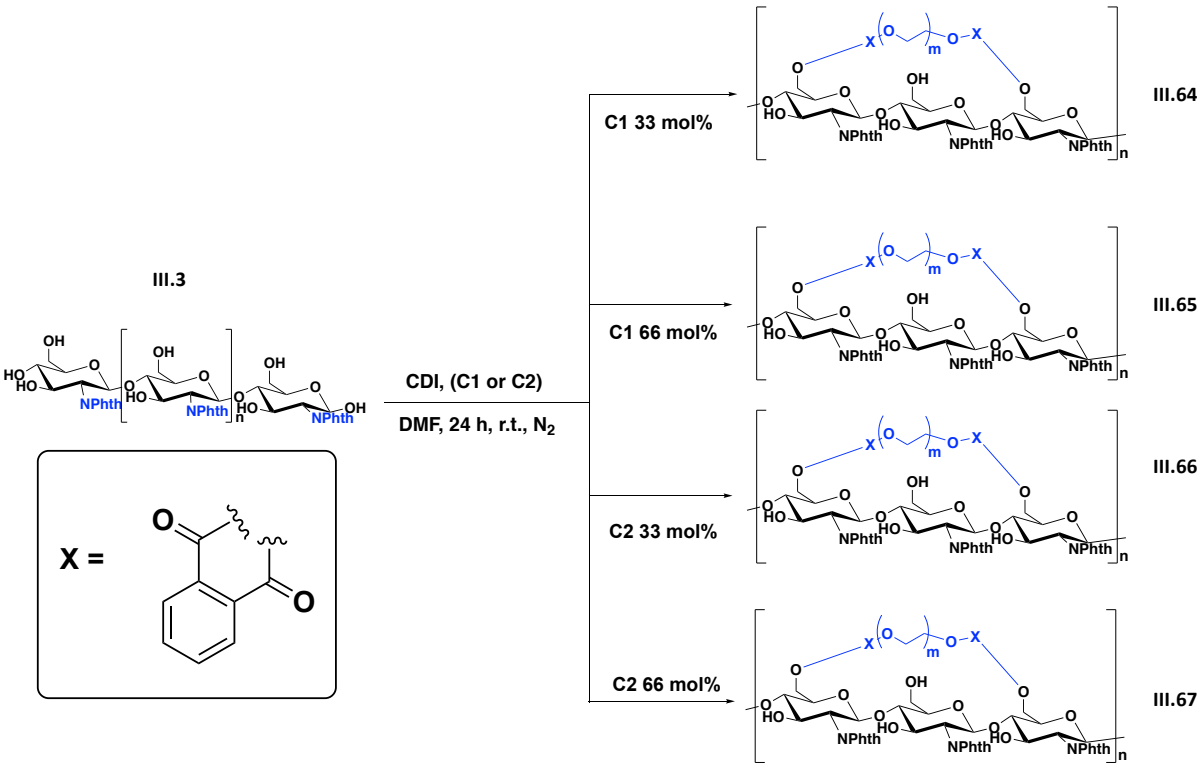
3.5.1.2 Preparation of dicarboxylic acids C1 and C2



Compound **C1,** to a solution of phthalic anhydride (15.0 mmol) in 100 mL of anhydrous DCM, was added the diethylenoglycol (5.0 mmol) and stirred for 10 minutes, under a nitrogen atmosphere. The reaction mixture was cooled to 0 ^{o}C and 15 mmol of anhydrous TEA was added drop-wise. The reaction was stirred overnight at room temperature. The solvent was evaporated, and the residue obtained cooled to 0 ^{o}C and 200 mL of saturated sodium bicarbonate solution was added. The aqueous layer was washed with ether, 3 x 100 mL. The aqueous layer was collected and cooled to 0 ^{o}C, acidified with diluted HCl 1 M and extracted with DCM (4 x 100 mL).[173] The combined organic layers were evaporated and dried under reduced pressure to provide a colourless oil in 90% yield. **^{1}H-NMR** (400 MHz; $CDCl_3$): δ 7.80-7.57 (m, 8H), 4.43 (t, *J* = 3.4 Hz, 4H), 3.77 (t, *J* = 3.3 Hz, 4H). **^{13}C NMR** (100 MHz; $CDCl_3$): δ 174.6, 167.7, 132.1, 131.8, 131.6, 131.4, 131.3, 130.8, 129.2, 128.7, 69.1, 65.8, 65.6. **FT-IR** ν_{max} (NaCl): 3054, 1726, 1702, 1172-1077 cm^{-1}. **HR-ESI:** 425.0895 [M+Na^{+}] (calculated), 425.0845 (found).

Compound **C2**, same procedure as **C1**, using triethylenoglycol instead of diethylenoglycol, affording a colourless oil in 63% yield. **^{1}H-NMR** (400 MHz; $CDCl_3$): δ 7.81-7.78 (m, 4H), 7.60-7.58 (m, 4H), 4.51 (dt, *J* = 4.0, 2.2 Hz, 4H), 3.88 (dt, *J* = 4.0, 2.2 Hz, 4H), 3.79 (s, 4H).**^{13}C-NMR** (101 MHz, $CDCl_3$): δ 171.5, 170.5, 167.6, 131.8, 131.7, 131.4, 131.3, 131.1, 129.3, 129.2, 129.1, 129.11, 73.8, 70.8, 70.2, 69.7, 68.9, 68.8, 64.6, 64.4. **HR-ESI:** 469.1111 [M+Na+] (calculated), 469.1108 (found).

3.5.1.3 Preparation of ester compounds III.64-67 from *N*-phthaloyl chitosan (III.3)



General procedure:

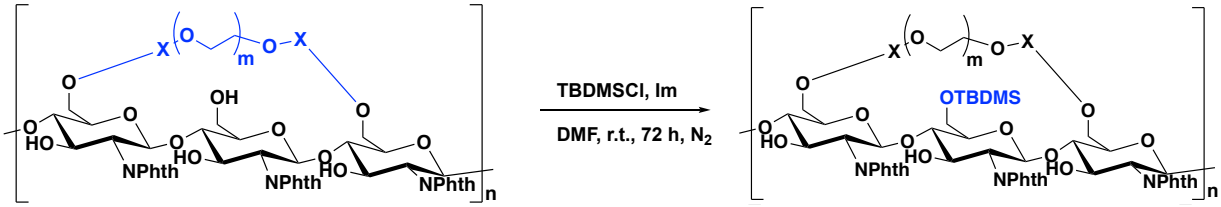
For compound **III.64**, the dicarboxylic acid **C1** (0.396 mmol, 0.33 equiv.), was dissolved in 3 mL of anhydrous DMF and with CDI was added (0.792 mmol) in, under a nitrogen atmosphere and the mixture was stirred at room temperature for 3 hours. The mixture was added drop-wise to a solution of **III.3** (300 mg, 1.2 mmol) and TEA (8.16 mmol) in 4 mL of anhydrous DMF, at room temperature under nitrogen atmosphere and the resulting mixture was stirred for 24 hours. The solvent was removed to dryness and the resulting crude washed t with 5 mL of methanol in an ice bath, to obtain **III.64**, 289 mg of a yellow solid. **^{13}C CP/MAS NMR** (101 MHz; None): δ 173.4 (C=O amide), 169.0 (C=O ester), 134.7, 130.7, 123.3 (Ar), 100.4 (C1), 82.4 (C4), 74.9 (C5), 70.3 (C3), 64.8 (-OCH_2), 61.1 (C6), 56.1 (C2). **FT-IR** ν_{max}(KBr): 3446, 2972, 2880, 1775, 1712, 1650, 1563, 1471, 1392, 1295, 1194-966.

Compound **III.65**: General procedure, using **C1** (0.792 mmol, 0.66 equiv.), **III.3** (300mg, 100%) CDI (1.584 mmol). Compound **III.65** was isolated as a yellow solid (320 mg). **^{13}C CP/MAS NMR** (101 MHz; None): δ 172.7, 168.7, 134.6, 130.4, 123.3, 100.3, 83.1, 74.7, 70.5, 65.4, 60.8, 55.9**.** **FT-IR** ν_{max} (KBr): 3428, 2924, 2860, 1771, 1387, 1289, 1196-974.

Compound **III.66**: General procedure, using **C2** (0.396 mmol, 0.33 equiv.), CDI (0.792 mmol). Compound **III.66** was isolated as a yellow solid (296.5 mg). **^{13}C CP/MAS NMR** (101 MHz, None): δ 254.8, 242.2, 168.6, 134.7, 130.9, 123.0, 100.6, 83.1, 75.0, 70.6, 60.8, 56.3, 22.2, 14.9, 11.8, 2.6. **FT-IR** ν_{max} (KBr): 3443, 2929, 2887, 1776,1712, 1662, 1560, 1550, 1469, 1391, 1321, 1197-970.

Compound **III.67**: General procedure, using **C2** (0.792 mmol, 0.66%), CDI (1.584 mmol). Compound **III.67** was isolated as a yellow solid (313 mg). **^{13}C CP/MAS NMR** (101 MHz, None): δ 255.1, 242.1, 168.9, 134.6, 130.6, 123.3, 100.4, 82.4, 74.7, 70.9, 61.0, 56.0, 22.5, 14.8, 10.9, 3.2. **FT-IR** ν_{max} (KBr): 3432, 2928, 2874, 1810, 1774, 1750, 1718, 1702, 1419, 1395, 1320, 1296, 1196-970.

3.5.1.4 Preparation of silylated esters III.68-71



III.68-III.71

General procedure

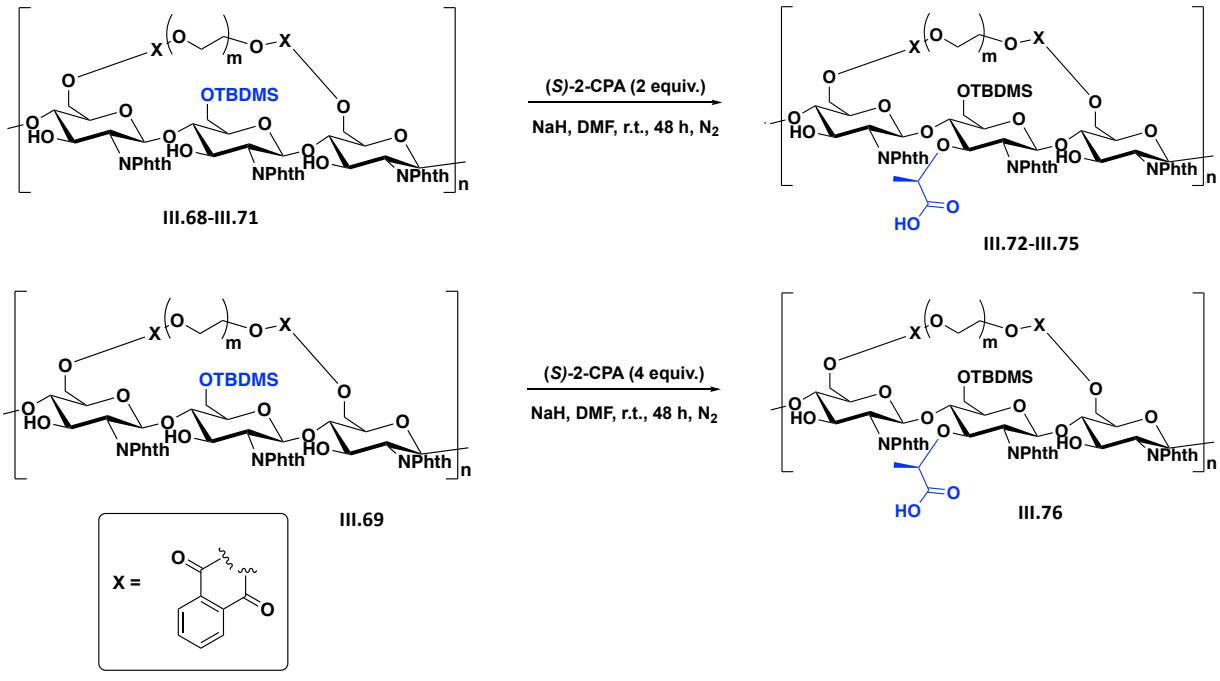
For compound **III.68**, to a suspension of **III.64** (290 mg, 1.05 mmol) and an excess of imidazole (8.53 mmol) in anhydrous DMF (40 mL) was treated with the TBDMSCl (4,97 mmol). The reaction was stirred at room temperature for 72 hours. The product was precipitated with 50 mL of water/ethanol (1:1 v/v) solution. Then the solid was filtered and washed with 20 mL of water/ethanol and ethyl ether 3 x 5 mL. Obtaining **III.68** 351 mg of a yellow solid.[140] **^{13}C CP/MAS NMR** (101 MHz, None): δ 168.2, 163.7, 134.5, 131.3, 123.8, 120.6, 99.5, 83.1, 75.2, 70.9, 63.0, 60.8, 56.1, 36.5, 31.2, 25.4, 22.7, 18.2, -5.9. **FT-IR** ν_{max} (KBr): 3460, 1755, 1718, 1664, 1467,1389, 1322, 1194-966, 1115, 1066.

Compound **III.69**: General procedure using **III.65**. Compound **III.69** was isolated as a yellow solid (396 mg). **^{13}C CP/MAS NMR** (101 MHz; None): δ 172.3 (C=O amide), 168.2 (C=O ester), 148.0, 134.4, 131.8, 123.6, 116.8 (Ar), 99.2 (C1), 83.2 (C4), 82.4 (-O- CH_2), 75.2 (C5), 71.0 (C3), 63.4 (C6), 55.9 (C2), 25.5 ($C(CH_3)_3$), -6.0 (CH3). **FT-IR** ν_{max} (KBr): 3466, 1778, 1729, 1646, 1471, 1390, 1326, 1196-970, 1119, 1071.

Compound **III.70**: General procedure using **III.66**. Compound **III.70** was isolated as a yellow solid (298 mg). **FT-IR** ν_{max} (KBr): 3448, 2930, 2884, 2858, 1776, 1717, 1663, 1468, 1390, 1322, 1255, 1196-969.

Compound **III.71**: General procedure using **III.67**. Compound **III.71** was isolated as a yellow solid (313 mg). **FT-IR** ν_{max} (KBr): 3467, 2951, 2929, 2884, 2857, 1779, 1717, 1533, 1471, 1387, 1318, 1291, 1243, 1173-969.

3.5.1.5 Insertion of lactate moiety - Preparation of compounds III.72-75



General Procedure

For compound **III.72**: To a (200 mg, 0.278 mmol) of **III.68** in 5 mL of anhydrous DMF was added NaH (3.8 mmol) at 0 ^{o}C and was stirred for 1 hour. Then the (*S*)-(–)-2-Chloropropionic acid was added (2 equiv.). The reaction was stirred from 0 ^{o}C to room temperature for 48 hours. Then the solvent was evaporated to dryness, and the product was diluted with 3 mL of distilled water and acidified with 1 M HCl to pH 3. The residues were washed with ethyl ether, 3 x 5 mL, and the aqueous layers were collected, and the solvent was evaporated. **III.72** was dried under vacuum, obtaining 218 mg of an orange solid. **FT-IR** ν_{max} (KBr): 3422, 2957, 2924, 2854, 1722-1640, 1458, 1259, 1208, 1090-990, 1070.

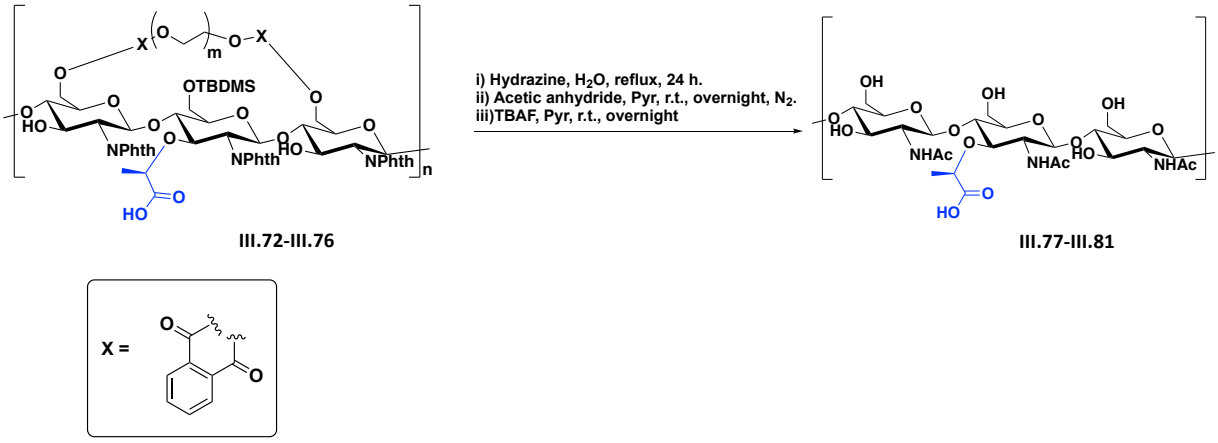
Compound **III.76**: General procedure using **III.69** and 4 equiv. of (*S*)-(–)-2-Chloropropionic acid. Compound **III.76** was isolated as an orange solid (239 mg). **FT-IR** ν_{max} (KBr): 3422, 2953, 2924, 2854, 1712-1609, 1458, 1399, 1148-988, 1081.

Compound **III.73**: General procedure using **III.69**. Compound **III.73** was isolated as an orange solid (324 mg). **FT-IR** ν_{max} (KBr): 3452, 2957, 2926, 2854, 2820, 1662-1578, 1454, 1398, 1353, 1241, 1151-986.

Compound **III.74**: General procedure, using **III.70**. Compound **III.74** was isolated as an orange solid (223 mg). **^{13}C CP/MAS NMR** (101 MHz): δ 174.3, 169.6, 134.0, 130.7, 101.4, 100.2, 74.3, 61.1, 55.7, 36.6, 35.5, 23.4, 17,8. **FT-IR** ν_{max} (KBr): 3448, 2950, 2930, 2859, 1774, 1718, 1663, 1587, 1438, 1391, 1277, 1259, 1227, 1169, 1106, 1063, 1033.

Compound **III.75**: General procedure using **III.71**. Compound **III.75** was isolated as an orange solid (190 mg). **^{13}C CP/MAS NMR** (101 MHz): δ 180.1, 174.0, 137.6, 129.3, 101.0, 74.8, 61.5, 55.2, 42.2, 36.8, 31.6, 26.1, 18.8, -4.8. **FT-IR** ν_{max} (KBr): 3443, 2955, 2925, 2855, 1671-1574, 1440, 1391, 1337, 1262, 1148-1021.

3.5.1.6 Removal of protecting groups and N-acetlyation - Preparation of compounds III.77-81



General Procedure

Compound **III.77**, to an aqueous solution of hydrazine monohydrate (40 equiv., 6 M) was added the corresponding substrate **III.72** (300 mg), and the mixture was heated to reflux for 24 h. The solvent was evaporated, and the residue washed with distilled water (5 mL), ethanol (5 mL) and ethyl ether (5 mL). The reaction was monitored by infra-red spectroscopy, and the product used directly in the next step without any further purification. To the previously prepared N-free chitosan derivate, acetic anhydride (20 equiv.) in dry pyridine (2 mL) at 0 ºC was added and the mixture stirred at room temperature overnight. Then the solvent was evaporated, and the crude obtained washed with ethyl ether, and to the crude obtained in 1 mL of distilled water and 0.1 mL of NaOH 1 M were added. The mixture was taken to dryness and the resulting residue dissolved in 1 mL of distilled water. The product was isolated by precipitation by addition of a solution of HCl 1 M till pH 3. The precipitated was filtered and dried. To the residue obtained a solution of TBAF 1 M in THF (2 equiv.) was added. The mixture was stirred overnight at room temperature. The solvent was removed, and the crude obtained was washed with ethyl ether (3 mL), and dissolved in distilled water (1 mL) and the pH adjusted to 3 with a solution of HCl 1 M till the product precipitated and it was filtered to give 110 mg of a light yellow solid. **III.77**: **^{13}C CP/MAS NMR** (101 MHz): δ 181.1, 174.2, 103.15, 82.7, 74.1, 61.0, 59.6, 55.0, 36.2, 22.9, 19.2. **FT-IR** ν_{max} (KBr): 3445, 2963, 2877, 1733, 1639, 1560, 1379, 1318, 1261, 1235, 1152-1031.

Compound **III.78**: General procedure using **III.74**, **III.78** was isolated as a light yellow solid (103 mg). **III.78**: **^{13}C CP/MAS NMR** (101 MHz): δ 181.0, 174.3, 102.6, 101.2, 82.6, 74.3, 60.1, 58.0, 54.6, 22.1, 19.6. **FT-IR** ν_{max} (KBr): 3446, 2964, 2876, 1639, 1562, 1413, 1340, 1157-1021.

Compound **III.79:** General procedure using **III.73**. Compound **III.79** was isolated as a light yellow solid (110). **III.79**: **^{13}C CP/MAS NMR** (101 MHz): δ 171.9 (carboxylic acid), 171.4 (amide), 103.2 (C1), 86.2 (C4), 73.3-72.7 (C3, C5), 63.6 (C6), 55.1 (C2), 22.7 (NCH_3), 20.6 (CH), 13.6 (CH_3). **FT-IR** ν_{max} (KBr): 3435, 1698, 1656, 1158-1012.

Compound **III.80**: General procedure using **III.75**. Compound **III.80** was isolated as a light yellow solid (98 mg). **III.80**: **^{13}C CP/MAS NMR** (101 MHz): δ 173.7, 171.8, 103.1, 85.8, 74.1, 61.7, 55.2, 22.9, 20.5, 13.7 **FT-IR** ν_{max} (KBr): 3429, 2964, 2876, 1741, 1650, 1562, 1487, 1469, 1377, 1306, 1243, 1151-1039.

Compound **III.81**: General procedure using **III.76**. Compound **III.81** was isolated as a light yellow solid (84 mg). **III.81**: **^{13}C CP/MAS NMR** (101 MHz): δ 174.5, 172.0, 103.1, 86.2, 76.7-74.3, 55.3, 22.8, 20.5, 13.7. **FT-IR** ν_{max} (KBr): 3429, 1717, 1651, 1635, 1151-1029.

3.5.2 Enzymatic digestion

For lysozyme digestion, the samples (10 mg/mL) in MilliQ H_2O were added to 10 mg/mL of lysozyme in ammonium acetate 40 mM, pH 5.25 at a final ratio of 1:0.225 [sample (mg): enzyme (mg)] and incubated for 92 h at 37 ^{o}C and 1200 rpm in an *eppendorf* dry block shaker. For mutanolysin digestion the samples (10 mg/mL) in MilliQ H_2O were added to 10 mg/mL of mutanolysin in 25 mM phosphate buffer at pH 5.5 at a ratio of 1:0.15 [sample (mg): enzyme (mg)] and incubated for 92 h at 37 ^{o}C and 1.200 rpm in an *eppendorf* dry block shaker. Enzymatic digestion was stopped by heat inactivation of the enzymes. The released material was collected as the supernatant after a 5 min centrifugation at 16000 g. free acetal ends Reduction of the samples were carried for 2 h at room temperature by mixing an equal volume of sample and 0.5 M Borate Buffer pH 9.0 and adding freshly prepared $NaBH_4$ at 50 mg/mL in MilliQ H_2O in a ratio of 8.33:1 (mixture (mL): $NaBH_4$ (mL)). The reduction reaction was stopped by lowering the pH to 2.0 with 85% *o*-phosphoric acid.

The profile of the resulting mixture of muropeptides was acquired by HPLC separation on a reverse phase C-18 Hypersil column as described.[180] The digested sample was separated on wide pore Nucleosil C18 reverse-phase HPLC column using a 0–10.5% acetonitrile convex gradient in 100 mM sodium phosphate buffer, pH 2, for 90 min followed by a 30% acetonitrile step in the same buffer. Peak detection was performed using a UV detector at 205 nm. The PGN fragments were obtained according to described procedure.[182]

Monitoring of the enzymatic digestion was carried by running the sample on the HPLC. A non-digested sample subjected to the same incubation conditions (time, temperature and buffer) was

used as negative control for the digested sample. HPLC analysis (*Appendix Section Figures S3-7*) best enzymatic digestion was achieved for sample **III.79**, which was further analysed by LC-MS (*Appendix Section Figure S3*). The efficiency of the enzymatic digestion was determined by careful analysis of LC-MS results (see Mass Spectrometry Section).

3.5.3 Monosaccharide composition analysis

The monosaccharide composition of the samples was determined by HPAEC-PAD as described.[183] 0.2 mg of sample was hydrolysed to monosaccharides by a 2 h incubation at 95 ºC in a 3 M HCl solution. The hydrolysis reaction was stopped by lyophilizing the HCl solution until dryness. The remaining HCl, which had not been evaporated, was diluted by adding 500 µL water and the resulting sample lyophilized until dryness. The released monosaccharides were resuspended on a final volume of 150 µL of MilliQ H_2O. Unless stated otherwise, 10 µL of the resulting samples were analysed on a CarboPac PA10 column following a previously described method.[182]

HPLC data: In order to test whether chitooligosaccharides released from unmodified chitin could be present in the digestion of synthetic sample, a control experiment was performed in which chitin was subjected to the same digestion conditions. Moreover, this control experiment allowed to investigate the selectivity of the enzymes for the modified sample.

According to the literature, lysozyme digestion of partially deacetylated chitin furnishes 33.1% of monosaccharide (N-acetylglucosamine) together with 26.3% of NAG-NAG and small amounts of higher oligosaccharides.[184]

Next, we investigated the digestion, under the same conditions, of PGN from *E. coli*. The PGN from *E. coli*[154] was used as a model to establish the optimum conditions for digestion as well as to test the HPLC program, column and conditions to follow the digestion of synthetic samples. These PGN samples contained the oligosaccharides and the peptide stems. The HPLC conditions were adapted from a literature procedure reported for PGN from *S. aureus.*[180]

3.5.4 Mass spectra

The LC–MS/MS analysis were performed on a Waters Alliance HPLC system (Waters, 2695 separation module, Ireland) comprising a quaternary pump, an on-line solvent degasser, autosampler and column oven. The separation of the compounds was done on a reversed-phase column (LiChrospher® 100 RP-18, 250x4 mm; 5 µm; Merck®) at 35 ºC using an injection volume of 20 µL. The mobile phase consisted of a Milli-Q water containing 0.5% formic acid (A): Acetonitrile (B). A flow rate of 0.30 mL/min was used, and the gradient conditions applied consisted of a linear increase from 100% to 0% (A) in 30 min; 20 min at 0% (A) and return to 100 % (A) in 10 min. The system was then re-equilibrated with 100% (A) for 20 min. Tandem mass spectrometry (MS/MS) detection was performed on a Micromass® Quattro Micro triple quadrupole (Waters®, Ireland) using an electrospray ionization (ESI) source operating at 120 ºC and applying a capillary voltage of 3.0 kV and cone voltage 30.0 V. High purity nitrogen (N_2) was used both as drying gas and as a nebulizing gas. Ultra-high purity argon (Ar) was used as collision gas and collision energy was 20-30 eV. MassLynx software (version 4.1) was used to control the system, for data acquisition and processing.

3.5.5 mCherry-PGRP-SA assays

Briefly 0.2 mg of PGN or the sample were incubated at 1200 rpm, 25 ^{o}C for 30 minutes on an Eppendorf mixer in 300 µL of a mCherry-PGRP-SA solution at 0.3 mg/mL in Phosphate Buffer Saline (PBS) pH 6.0. The unbound fraction was recovered by collecting the supernatant after centrifugation at 3000 rpm for 10 min. The bound fraction (pellet) was washed twice with 300 µL of PBS and recovered by centrifugation at 6000 rpm for 5 min and 12000 rpm for 3 minutes respectively. A reaction with no peptidoglycan added was used as a protein precipitation control.

3.5.6 Quantification studies

In order to quantify the amount of each sample digested, the peak observed at 870 m/z from the TCI of the non-digested sample was used as reference (corresponding to the non-digested polymer). This peak was also observed in the LC-MS of sample **III.79,** corresponding to the non-digested polymer (45% of the initial). Using the calibration curve (*Appendix Section Figure S12*) was possible to calculate the amount of disaccharide present in the digested polymer. The NAG-NAM disaccharide was synthesized according to the reported procedure.[181]

3.6 References

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4 General Conclusions

Carbohydrates, the most abundant class of organic compounds in nature, are essential components of the cell surface of both bacteria and mammalian cells, and are involved in biological phenomena such as cell-cell communication and pathogen infection.

Peptidoglycan (PGN), also known as murein, the major component of the bacterial cell wall, is made of repeating *N*-acetylglucosamine (GlcNAc or NAG)–*N*-acetylmuramic (MurNac or NAM) disaccharide units, linked via [NAG-(β-1,4)-NAM] linkage, with stem peptides attached to the D-lactyl (Lac) moiety of each NAM.

The modification of the composition and synthesis of the PGN in different bacteria is associated with expression of bacterial resistance to different antibiotics and with a variety of host/bacteria interactions. The study of the relevance of the PGN structure in the metabolism of bacteria and the determination of its role in host disease has been hampered by the lack of pure PGN fragments from natural sources, and the need of ligand purification in reliable amounts. Indeed, the extraction and purification process of PGN presents several difficulties, due to the presence of teichoic acids and proteins that can contaminate the PGN fragments. Thus, several groups have been dedicated to the development of novel synthetic strategies to access the complex and unique PGN structure.

Synthesis of NAG containing oligosaccharides, such as NAG-NAM disaccharide, is a challenging task due to the well-known limitations associated with the enantioselective glycosylation of NAG moieties: i) the presence of a β-(1,4) glycosidic bond requires a multi-step synthetic sequence to obtain a regio- and stereoselective assembly of glycosidic bonds, crucial for biological activity; ii) the use of 2-acetamido-2-deoxyglycosyl donors, due to the formation of 1,2-O,N-oxazoline intermediates, and the use of corresponding acceptors that are poor nucleophiles.

Organic chemists are inspired by nature and have been developing strategies to synthesize PGN fragments similar to those used by bacteria to synthesize PGN. The similarity between both approaches, from bacteria and synthetic, can be summarized and highlighted by some key steps: anomeric activations, which will allow further glycosylation in order to elongate the carbohydrate chain; NAG O-3 alkylation, to install the Lac moiety converting NAG units into NAM units; peptide coupling at the O-3 Lac unit and a final glycosylation step, Figure 4.1.

In this work the strategy was to innovate the synthesis of PGN fragments. Since one of the major difficulties is the establishment of the β-(1,4) glycosidic bond, a new route was investigated using a biopolymer that contains the desired glycosidic bond: chitin and chitosan.

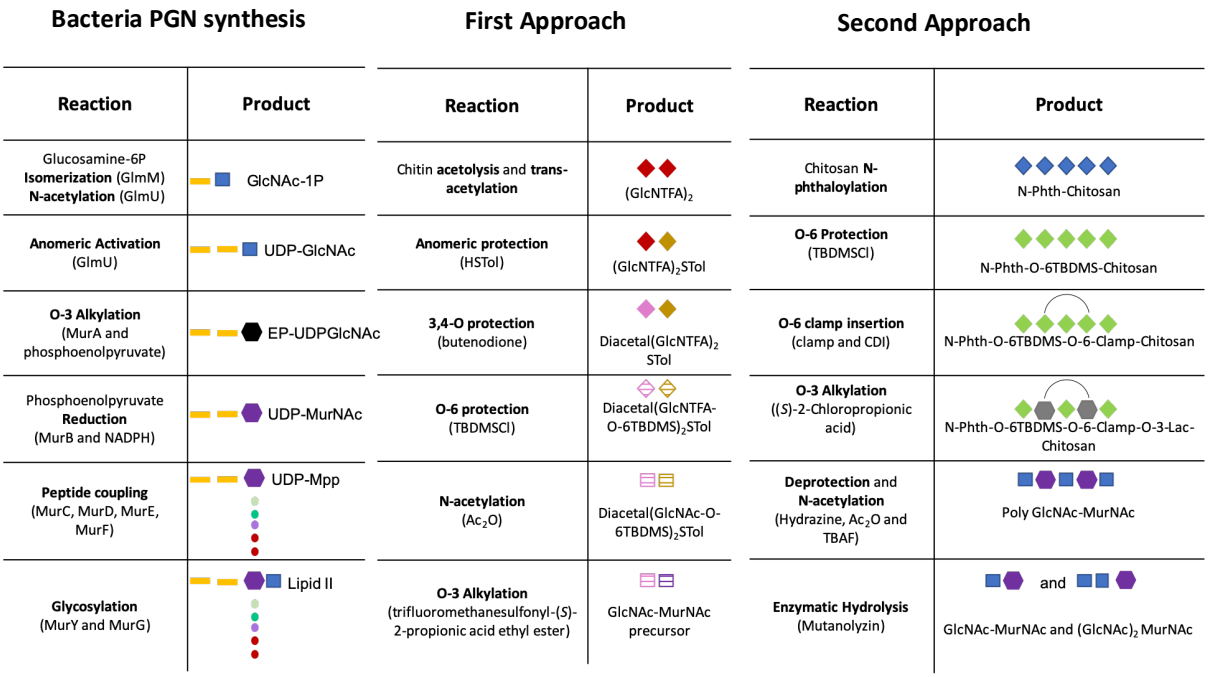

Figure 4.1 PGN synthetic strategies summary.

Two different approaches were developed in this Thesis, both from biomass chitosan. Preliminary experiments were focused on the regioselective modification of chitobiose derivatives or glucosamine derivatives, via selective oxidation or replacement of the N-acetyl group by other N-protecting groups, that would enable further modification of the carbohydrate chain to access NAG-NAM oligosaccharides. On the basis of the results obtained, two strategies were designed that led to the preparation of an advanced intermediate towards NAG-NAM disaccharide and NAG-NAM containing oligosaccharides, Figure 4.2. The first approach involved the use of peracetylated chitobiose as staring material, that was obtained in gram scale from the acetolysis of chitin. Several experiments were carried to establish the best synthetic route, optimize the protecting groups for both the NH2 and OH groups. A NAG-NAM precursor, a key intermediate to achieve NAG-NAM oligosaccharides was prepared in five steps, with an overall yield of 4.2 %. The *trans*-N-acetylation of the chitobiose derivative to NTFA revealed to be crucial as well as the combination of the different O-protecting groups, that allowed the regioselective

introduction of the critical lactyl unit at O-3. The regioselective protection of O-3 and O-4 of the terminal residue represented a challenge that was overcome by the use of the butadione group. This protocol takes advantage of the already installed β-1,4 glycosydic bond, present in chitobiose, which constitutes the main bottleneck of traditional approaches. This simple approach provides a fast access to a versatile intermediate, Figure 4.2, from a gram-scale peracetylated disaccharide.

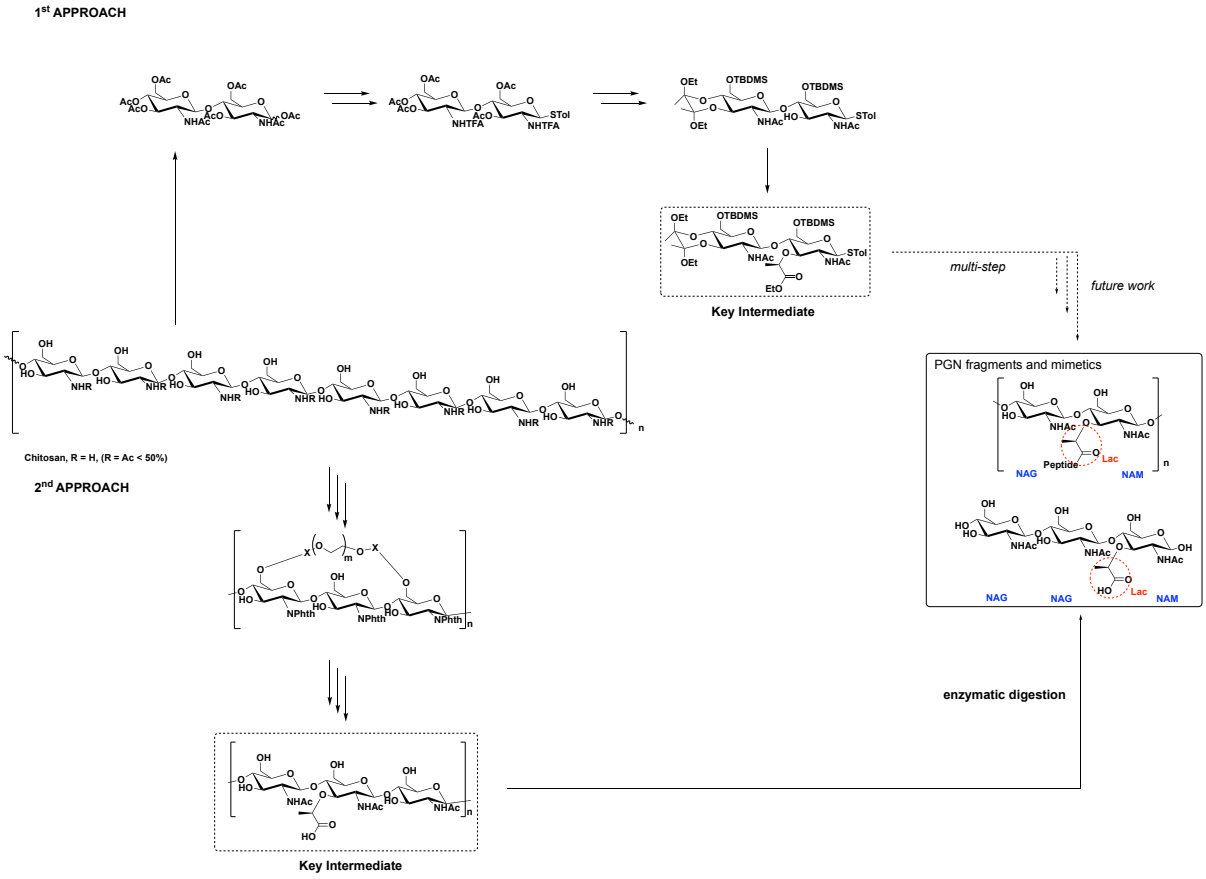

Figure 4.2 Summary of the two approaches investigated towards NAG-NAM precursors and mimetics

Chitosan was explored as a reliable template for a chemo-enzymatic approach towards NAG-NAM containing oligosaccharides, Figure 4.2 – the second approach. In this approach, the novel bacterial cell wall surrogates were obtained from chitosan via an unprecedented top down approach involving both chemical and enzymatic reactions.

Thus, chitosan was chemically modified, via N-protection and a molecular clamp was introduced at O-6 to differentiate the access to O-3 positions, via regio and steric discrimination. Two different carboxylic acids were used and the molecular clamp based

strategy allowed the productions of NAG-NAM containing oligosaccharides. The intercalation of the generated NAM residues was

confirmed through the analysis of oligosaccharide fragments released upon enzymatic digestion by mutalolysin and lysozyme, and the composition of the final polymer was analyzed by different techniques. NAG-NAM containing oligosaccharides were prepared in 33% yield from chitosan in eight steps. The fragments obtained

mimic the carbohydrate basic skeleton of most bacterial cell surfaces and were recognized by different molecular recognition systems, including PGRP-SA.

The compounds produced are biologically relevant and allow further studies in the glycobiology field. These studies open room for improvement in the access to such complex structures and on the discovery of key interactions between host and bacteria, crucial to understand bacterial infections.

All the work developed in this Thesis, the compounds prepared, and the precursors achieved enable further molecular recognition studies with different receptors and enzymes of both bacterial cell wall and innate immune system.

These strategies provide a simple route for an easy access to bacterial cell wall fragments – biologically important targets.

5 Appendix

5.1 HPLC data

In order to understand if chitooligosaccharides released from unmodified chitin could be present in the digestion of synthetic sample, a control experiment was performed in which chitin was subjected to the same digestion conditions. Moreover, this control experiment allowed to investigate the selectivity of the enzymes for the modified sample.

According to the literature, lysozyme digestion of partially deacetylated chitin furnishes 33.1% of monosaccharide (N-acetylglucosamine) together with 26.3% of NAG-NAG and small amounts of higher oligosaccharides.[8]

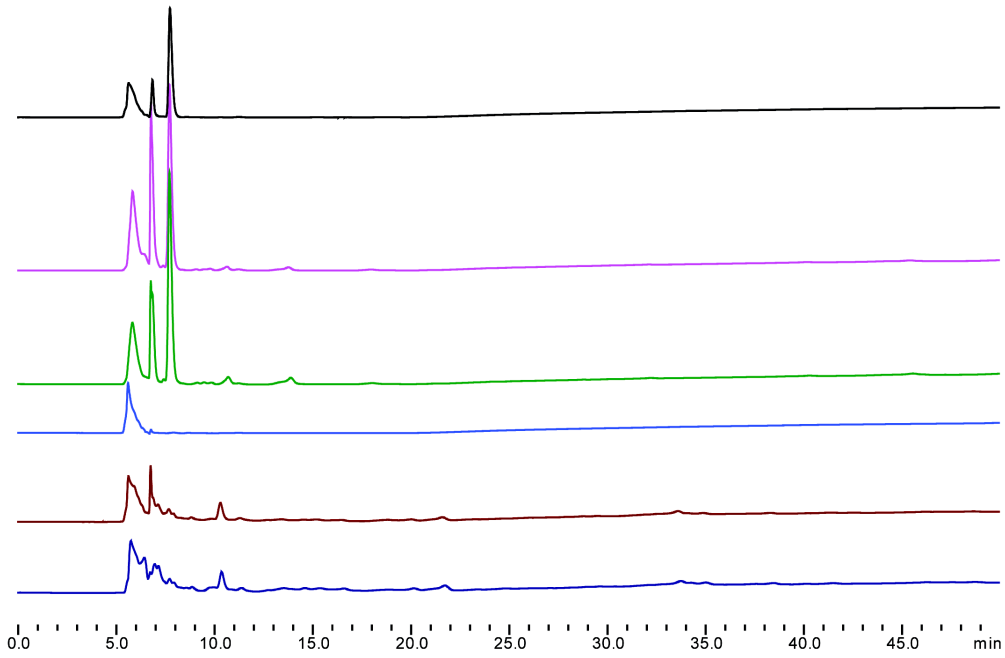

Figure S1: Chitin; Lysozyme negative control (under the same conditions: buffer, temperature and reaction time without enzyme) – black; Lysozyme digestion – purple; Lysozyme buffer positive control – green; Mutanolysin negative control (under the same conditions: buffer, temperature and reaction time without enzyme) – light blue; Mutanolysin digestion – brown; Mutanolysin positive control – dark blue.

Next, we investigated the digestion, under the same conditions, of PGN from *E. coli*. The PGN from *E. coli* was used as a model to establish the optimum conditions for digestion as well as to test the HPLC program, column and conditions to follow the digestion of synthetic samples. These PGN samples contained the oligosaccharides and the peptide stems.

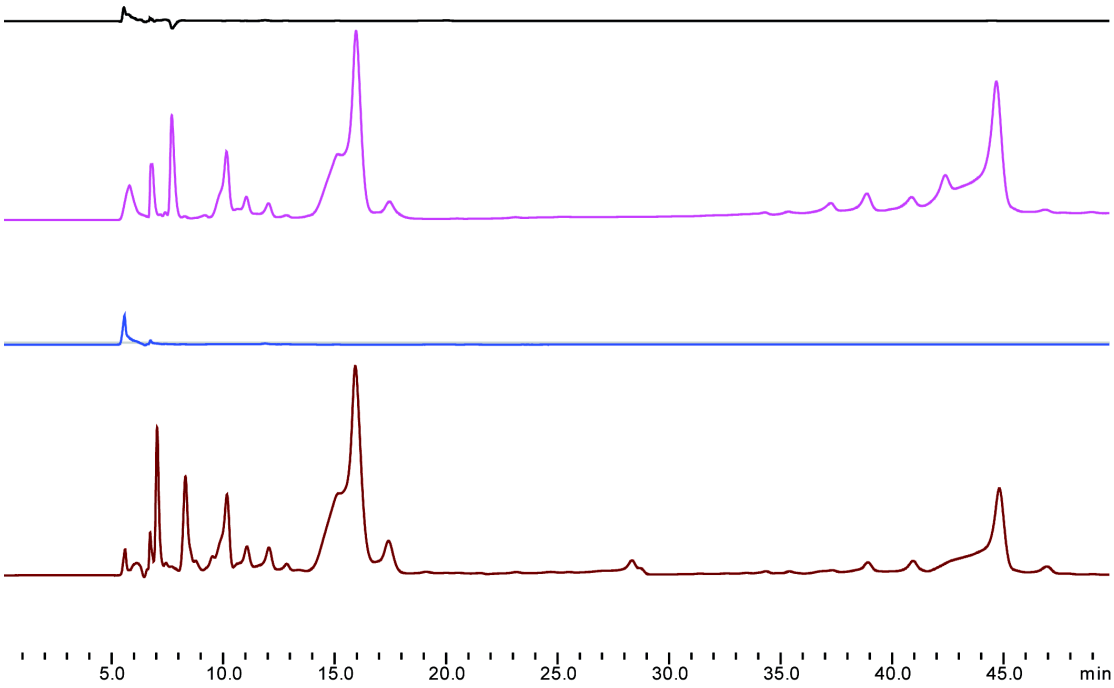

Figure S2: PGN; Lysozyme negative control (under the same conditions: buffer, temperature and reaction time without enzyme – black; Lysozyme digestion – purple; Mutanolysin negative control (under the same conditions: buffer, temperature and reaction time without enzyme) – blue; Mutanolysin digestion – brown.

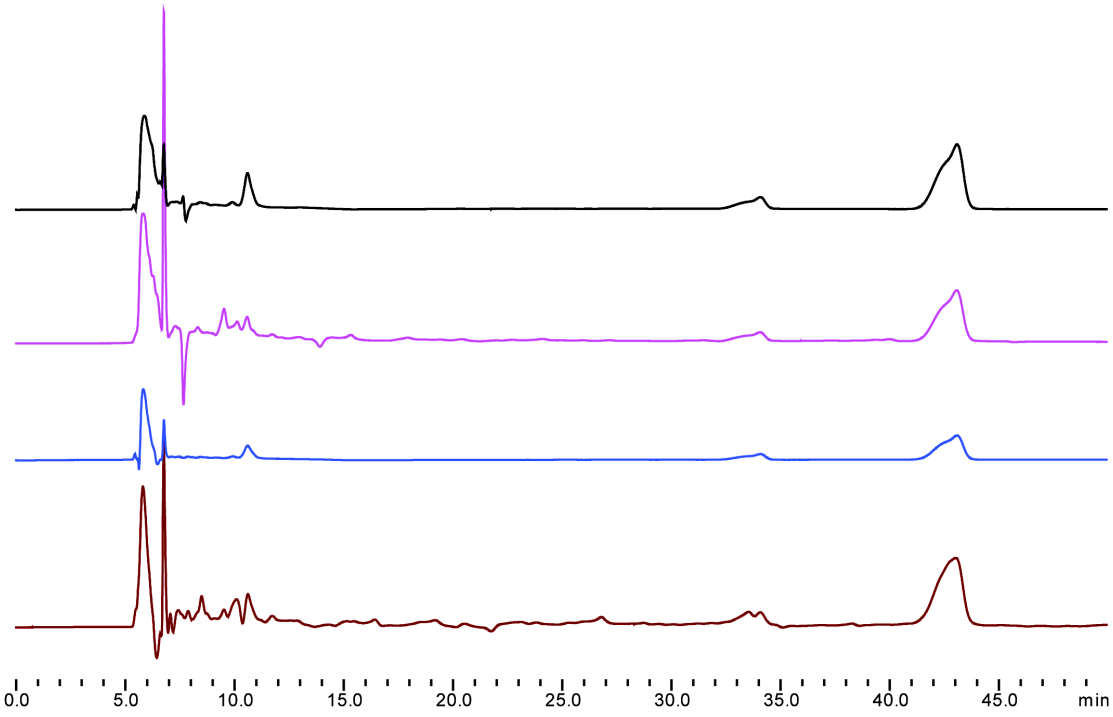

Figure S3: III.79; Lysozyme negative control (under the same conditions: buffer, temperature and reaction time without enzyme) – black; Lysozyme digestion – purple; Mutanolysin negative control (under the same conditions: buffer, temperature and reaction time without enzyme) – blue; Mutanolysin digestion – brown.

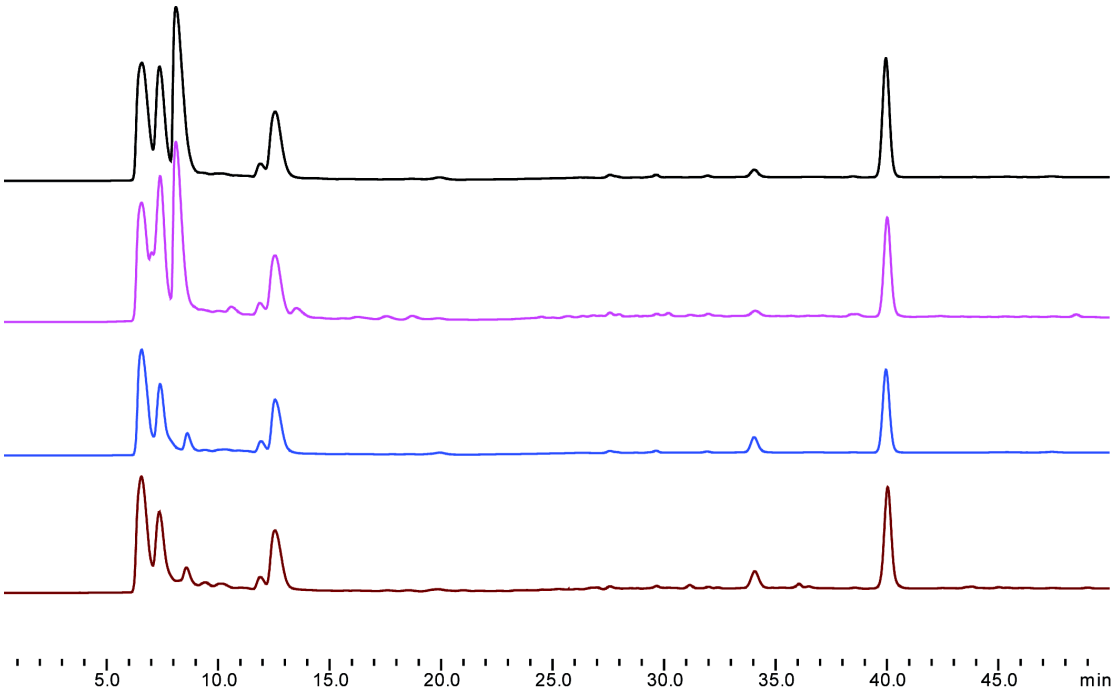

Figure S4: III.81; Lysozyme negative control (under the same conditions: buffer, temperature and reaction time without enzyme) – black; Lysozyme digestion – purple; Mutanolysin negative control (under the same conditions: buffer, temperature and reaction time without enzyme) – blue; Mutanolysin digestion – brown.

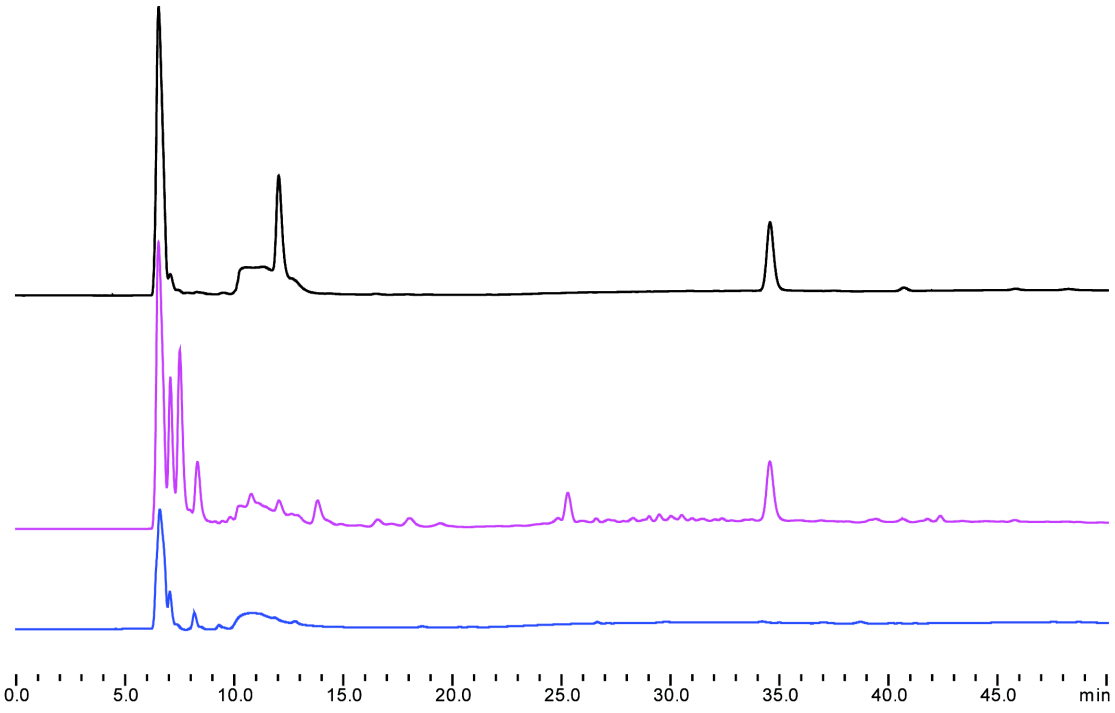

Figure S5: III.77; Lysozyme digestion– black; Mutanolysin digestion– purple; Negative control (under the same conditions: buffer, temperature and reaction time without enzyme) – blue.

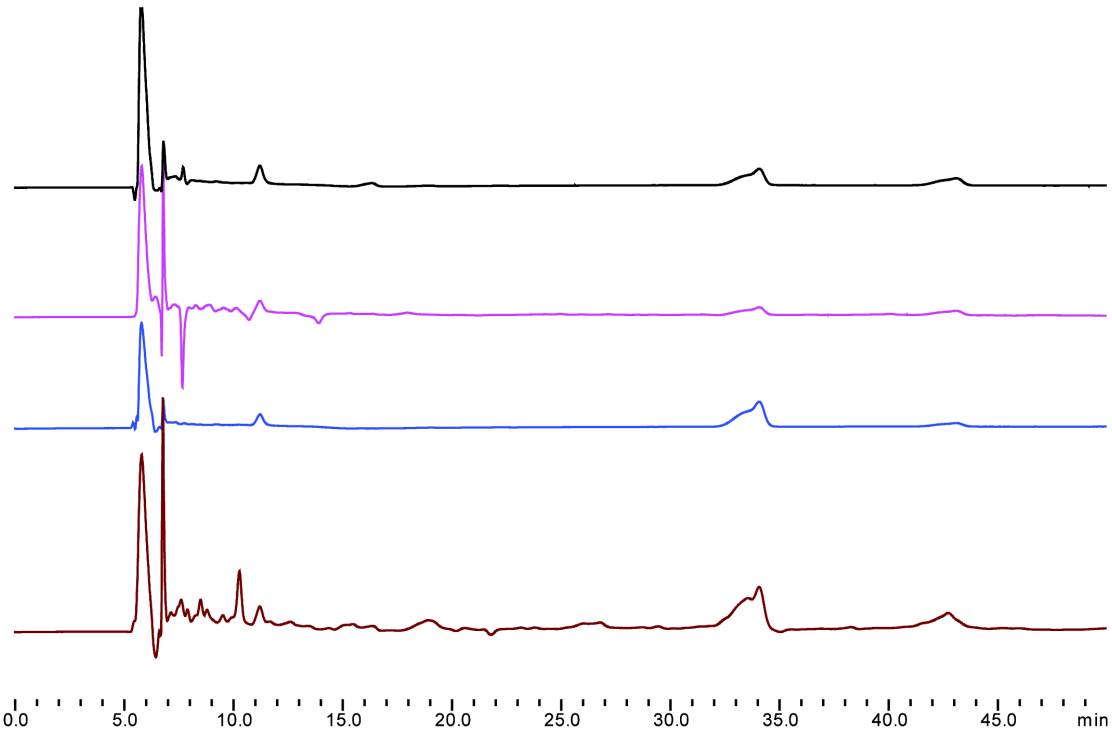

Figure S6: III.80; Lysozyme negative control (under the same conditions: buffer, temperature and reaction time without enzyme) – black; Lysozyme digestion – purple; Mutanolysin negative control (under the same conditions: buffer, temperature and reaction time without enzyme) – blue; Mutanolysin digestion – brown.

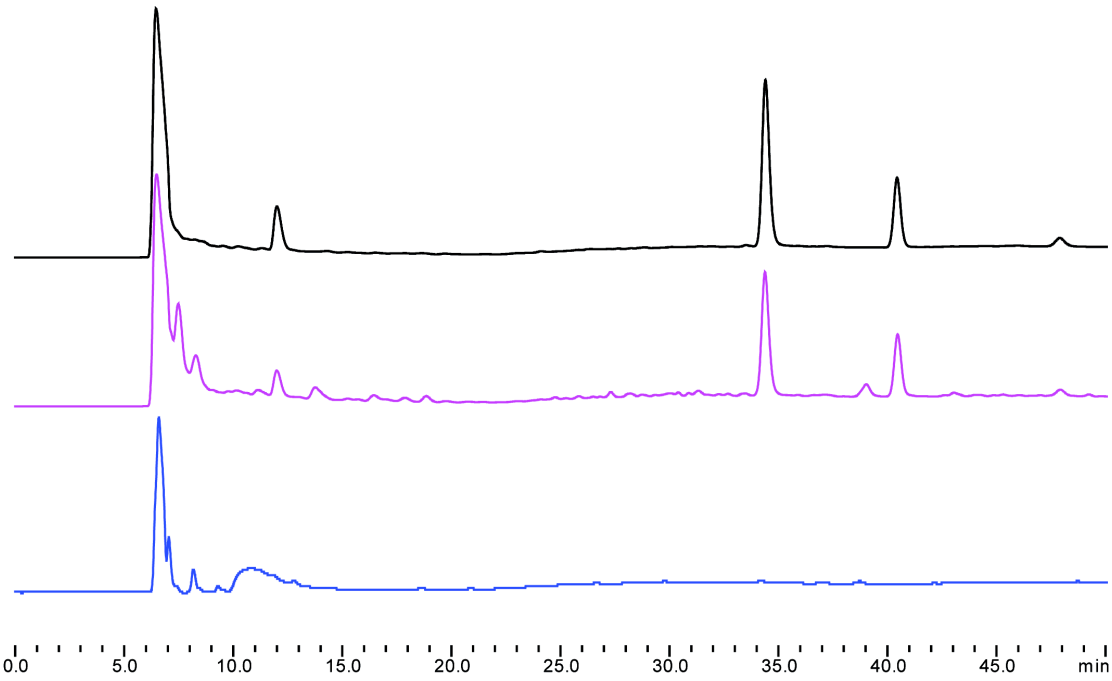

Figure S7: III.78; Lysozyme digestion– black; Mutanolysin digestion– purple; Negative control (under the same conditions: buffer, temperature and reaction time without enzyme) – blue.

5.2 LC-MS data

The LC–MS/MS analysis were performed on a Waters Alliance HPLC system (Waters_, 2695 separation module, Ireland) comprising a quaternary pump, an on-line solvent degasser, autosampler and column oven. The separation of the compounds was done on a reversed-phase column (LiChrospher® 100 RP-18, 250x4mm; 5□m; Merck®) at 35 ºC using an injection volume of 20 µL. The mobile phase consisted of a Milli-Q water containing 0.5% formic acid (A): Acetonitrile (B). A flow rate of 0.30 mL/min was used, and the gradient conditions applied consisted of a linear increase from 100% to 0% (A) in 30 min; 20 min at 0% (A) and return to 100 % (A) in 10 min. The system was then re-equilibrated with 100% (A) for 20 min. Tandem mass spectrometry (MS/MS) detection was performed on a Micromass® Quattro Micro triple quadrupole (Waters®, Ireland) using an electrospray ionization (ESI) source operating at 120 ºC and applying a capillary voltage of 3.0 kV and cone voltage 30.0 V. High purity nitrogen (N_2) was used both as drying gas and as a nebulizing gas. Ultra high-purity argon (Ar) was used as collision gas and collision energy was 20-30 eV. MassLynx software (version 4.1) was used to control the system, for data acquisition and processing.

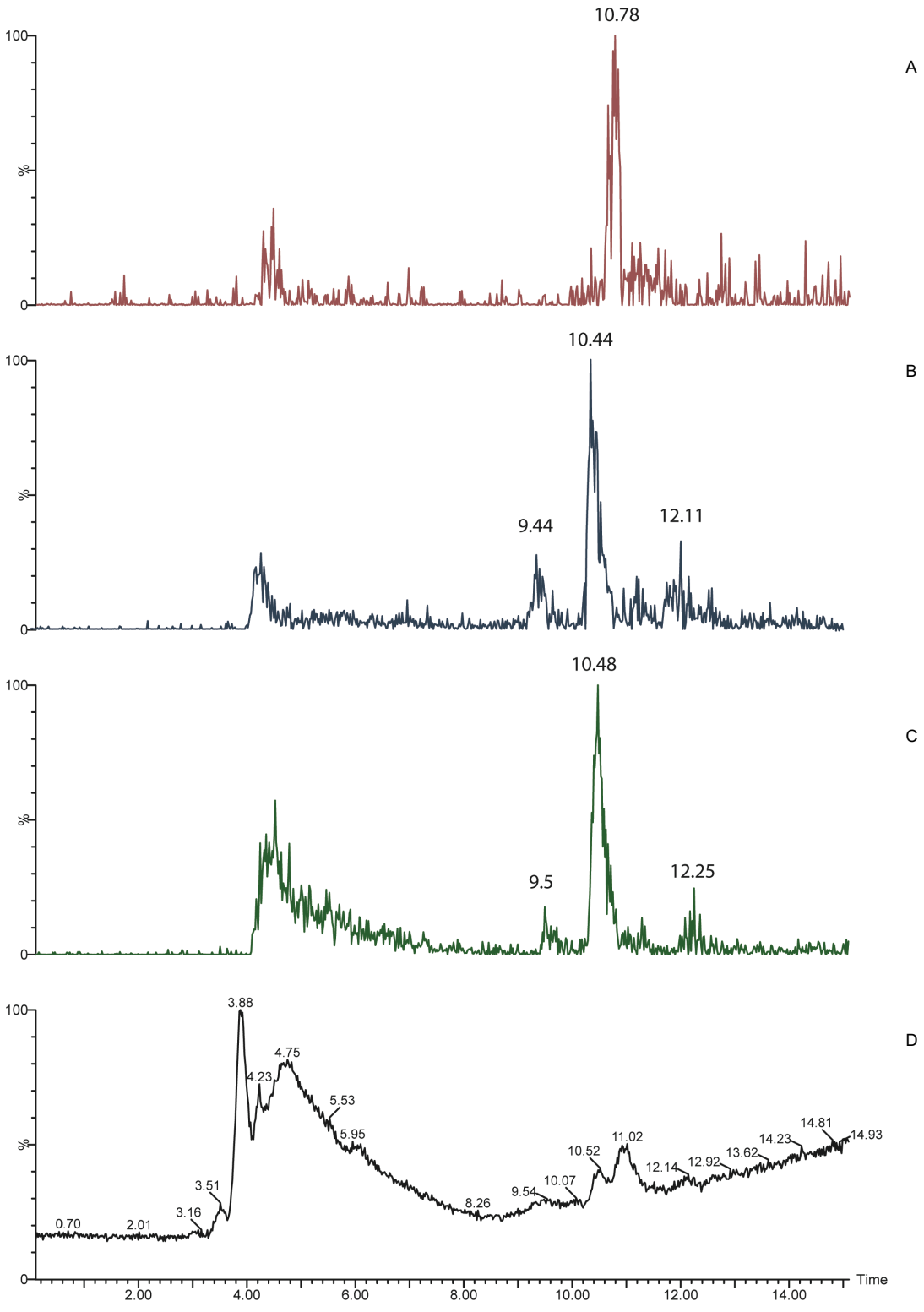

Figure S8: III.79 chromatogram after mutanolysin digestion. (A) 724 m/z detection mode; (B) 499 m/z detection mode; (C) 521 m/z detection mode; (D) LC/MS TIC.

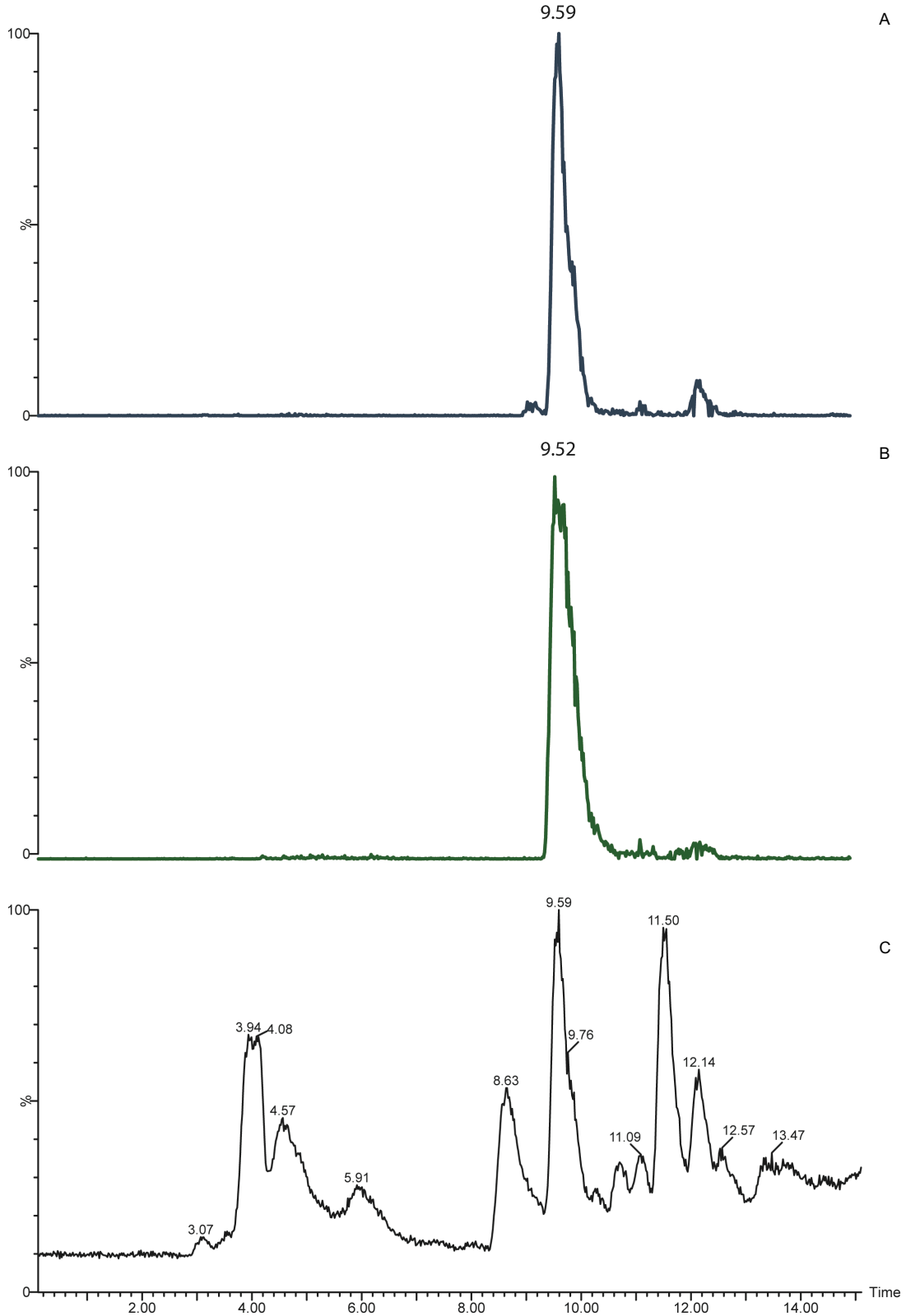

Figure S9: E. coli chromatogram after mutanolysin digestion. (A) 499 m/z detection mode; (B) 521 m/z detection mode; (C) LC/MS TIC.

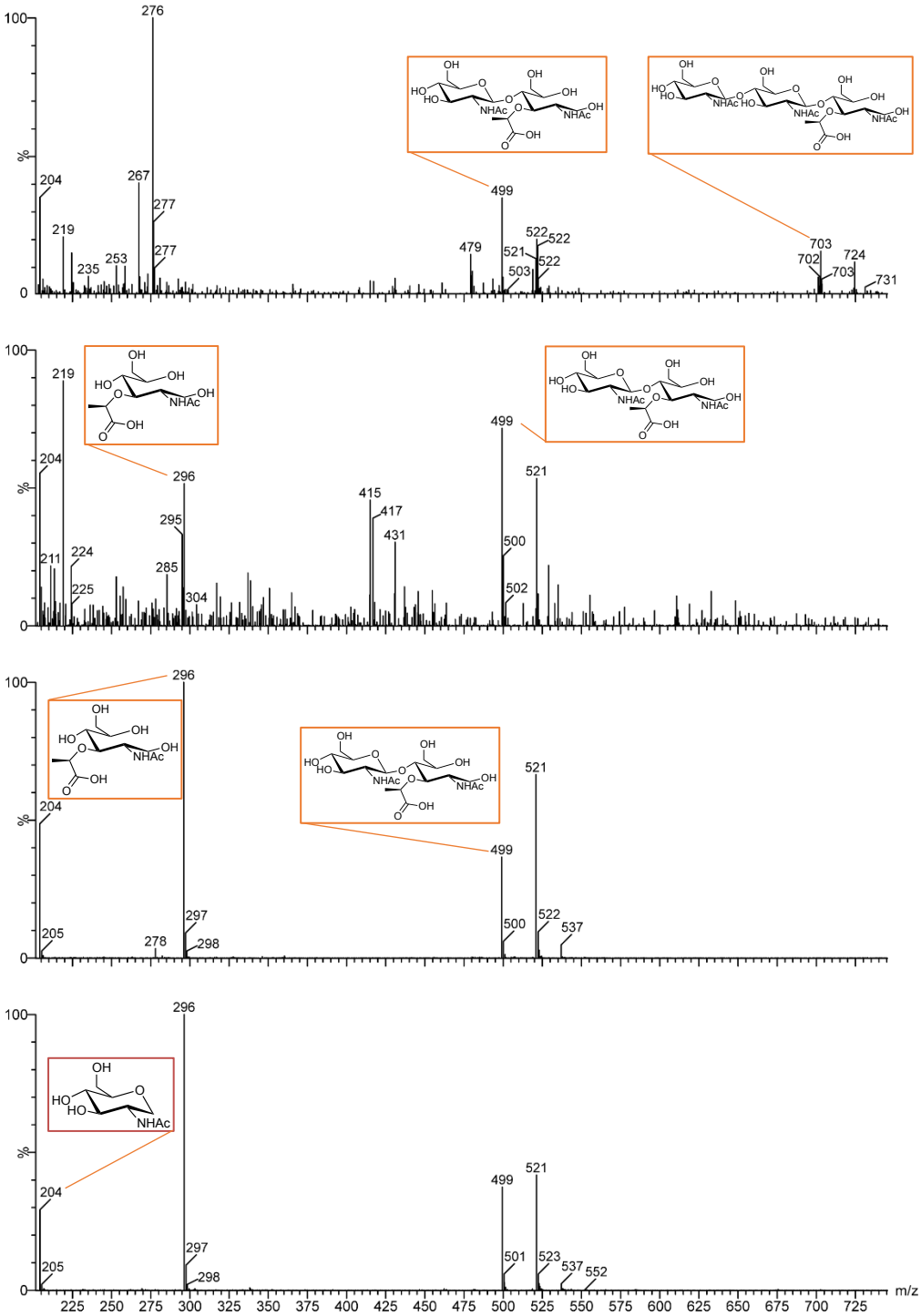

Figure S10: ESI mass spectra. (A) III.79 at 10.74 min; (B) III.79 at 9.50 min; (C) Synthetic NAG-NAM [8]; (D) E. coli at 9.57 min.

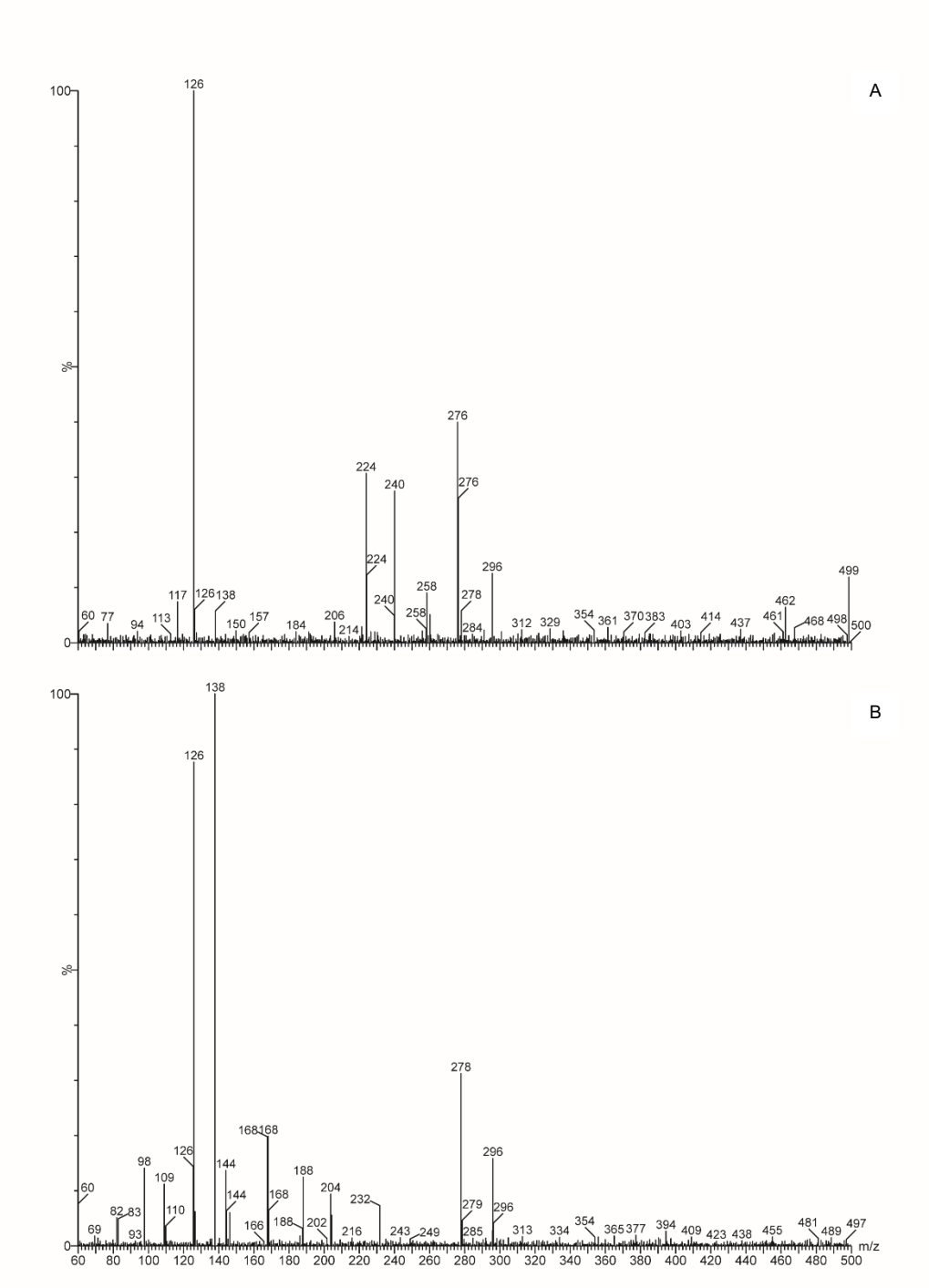

Figure S11: MS/MS mass spectra. (A) III.79; (B) PGN from E. Coli.

5.3 Quantification studies

The LC–MS/MS analysis were performed on a Waters Alliance HPLC system (Waters_, 2695 separation module, Ireland) comprising a quaternary pump, an on-line solvent degasser, autosampler and column oven. The separation of the compounds was done on a reversed-phase column (LiChrospher® 100 RP-18, 250x4mm; 5□m; Merck®) at 35 ºC using an injection volume of 20 µL. The mobile phase consisted of a Milli-Q water containing 0.5% formic acid (A): Acetonitrile (B). A flow rate of 0.30 mL/min was used, and the gradient conditions applied consisted of a linear increase from 100% to 0% (A) in 30 min; 20 min at 0% (A) and return to 100 % (A) in 10 min. The system was then re-equilibrated with 100% (A) for 20 min. Tandem mass spectrometry (MS/MS) detection was performed on a Micromass® Quattro Micro triple quadrupole (Waters®, Ireland) using an electrospray ionization (ESI) source operating at 120 ºC and applying a capillary voltage of 3.0 kV and cone voltage 30.0 V. High purity nitrogen (N2) was used both as drying gas and as a nebulizing gas. Ultra high-purity argon (Ar) was used as collision gas and collision energy was 20-30 eV. MassLynx software (version 4.1) was used to control the system, for data acquisition and processing. The NAG-NAM disaccharide was synthesized according to the reported procedure.[9]

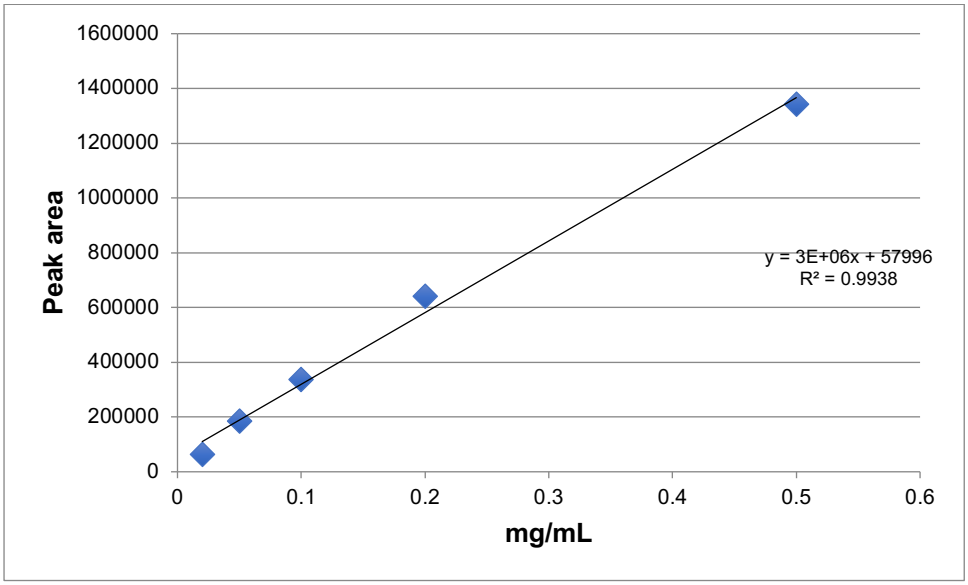

Figure S12: Calibration curve using synthetic NAG-NAM unit

Table S1: Quantification of sample III.79 (TCI in Figure S26 B) using Figure S12 calibration curve

Retention time (min)	9.46	10.48	11.27	12.14
Peak area	97112	323731	89494	179117
mg/mL	0,026077333	0,177156667	0,020998667	0,080747333

For the quantification of the total digested amount, the only peak observed was 870 m/z from the TCI of the non-digested sample was used as reference (corresponding to the non-digested polymer). The LC-MS of sample **III.79** showed also a peak of 870 m/z, corresponding to the non-digested polymer (45% of the initial). Using the calibration curve (described above) the amount of disaccharide was calculated for the LC-MS peak of correspondent mass.